

EXHIBIT 6



Europäische Patentamt
European Patent Office
Office européen des brevets



(11) Publication number: 0 128 332 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification :
02.08.95 Bulletin 95/31

(51) Int. Cl.⁶ : G01N 33/53, C07H 21/00

(21) Application number : 84105028.9

(22) Date of filing : 04.05.84

(54) Assay method utilizing polynucleotide sequences.

Divisional application 94102973.8 filed on
04/05/84.

(30) Priority : 05.05.83 US 491929

(43) Date of publication of application :
19.12.84 Bulletin 84/51

(45) Publication of the grant of the patent :
02.08.95 Bulletin 95/31

(84) Designated Contracting States :
AT BE CH DE FR GB IT LI LU NL SE

(56) References cited :
US-A- 4 358 535
JOURNAL OF MOLECULAR BIOLOGY, vol.
113, June 15 - July 15, 1977, London, New York,
San Francisco P.W.J. RIGBY et al. "Labeling
Deoxyribonucleic Acid to High Specific Activity
In Vitro by Nick Translation with DNA Polymerase 1" pages 237-251
PROCEEDINGS OF THE NATIONAL ACADEMY
OF SCIENCES OF THE UNITED STATES
OF AMERICA, vol. 78, no. 11, November 1981
P.R. Langer et al. "Enzymatic
syntheses of biotinlabeled polynucleotides:
Novel nucleic acid affinity probes" pages
6633-6637

(56) References cited :
NATURE, vol. 265, February 1977 G.T. RUDKIN
and B.D. STOLLAR "Highresolution detection
of DNA-RNA hybrids in situ by indirect im-
muno-fluorescence" pages 472-473
CHROMOSOMA; VOL 53, 1975, Berlin, Heid I-
berg, New York J.E. MANNING et al."ANew
Method of in situ Hybridization" pages
107-177

(73) Proprietor : ENZO BIOCHEM, INC.
325 Hudson Street
New York, N.Y. 10013 (US)

(72) Inventor : Pergolizzi, Robert G.
375 New Bridge Road
New Milford New York (US)
Inventor : Stavrianopoulos, Jannis G.
515 West 59 Street
New York New York (US)
Inventor : Rabbani, Elazar
69 Fifth Avenue
New York New York (US)
Inventor : Engelhardt, Dean L.
173 Riverside Drive
New York New York (US)
Inventor : Kline, Stan
235 Lincoln Place
Brooklyn New York (US)

(74) Representative : VOSSIUS & PARTNER
Postfach 86 07 67
D-81634 München (DE)

EP 0 128 332 B1

Note : Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid (Art. 99(1) European patent convention).

D scripti n

The present invention relates to assays, both immunoassays and nucleic acid assays of analytes, which utilize a universal detection system based on polynucleotide interactions.

The analysis and detection of minute quantities of substances in biological and non-biological samples has become a routine practice in clinical and analytical laboratories around the world. Broadly, the analytical techniques can be divided into those based on ligand-receptor interactions (e.g., immunoassay-based techniques), and those based on nucleic acid hybridization (polynucleotide sequence-based techniques).

For example, immunoassay techniques involve, at some stage or step in the process, the non-covalent association between an antibody binding site and an antigen complementary thereto. See, for example, "An Introduction to Radioimmunoassay and Related Techniques" by T. Chard, North Holland Publishing Company, Amsterdam, New York, Oxford, 1978. In polynucleotide sequence-based techniques, the process, at some step or another, involves the non-covalent binding of a polynucleotide sequence to a complementary sequence under hybridization conditions. (See for example, Falkow et al., U.S. Patent 4,358,535, Wahl et al., U.S. Patent 4,302,204, and Heimer, U.S. Patent 3,755,086.)

EP-A-0 124 221 which is a document according to Art. 54(3) and (4) EPC discloses a diagnostic method involving the binding of 3 entities to form a complex, followed by the detection of the signal whereby the three entities are the following:

- (1) the analyte, i.e., a nucleotide sequence to be detected;
- (2) a bridging entity comprising a nucleic acid sequence complementary to that which is to be detected and a polynucleotide tail, preferably a poly d(A)- or poly (dT)-tail and
- (3) a signal entity or marker comprising a polynucleotide capable of complexing to the polynucleotide portion of said bridging entity and a signal generating portion wherein an enzyme (HRPO) is attached to a single stranded portion of the polynucleotide.

In a generalized sense, both the aforementioned techniques involve a primary recognition event, brought about by precise molecular alignment and interaction, and energetically favored by the release of non-covalent bonding free energy (e.g., hydrogen bonding, dispersion bonding, ionic bonding, dipolar bonding, and the like). In addition to the primary recognition event, both techniques also involve, at one step or another, a signalling event. This step or event relates to the necessity of detecting, in some demonstrable manner to a human or instrument detection system, the primary recognition event.

Signalling has been centered mainly in two broad

areas: radioactive and non-radioactive techniques. Radioactive signalling has relied on radiolabeling of one or more components involved in the system, with such atoms as ^{32}P , ^{131}I , ^{14}C , ^3H , and the like. Detection is usually by means of a radioactivity detector. Non-radioactive techniques have been increasingly used in the last few years, since they involve no radioactivity, thus making such techniques safer, cleaner and more stable towards storage. They have been developed to sensitivities as high if not higher than radiolabeling techniques. Among the most common non-radioactive signalling techniques used at present are enzyme linked immunoassays (see, for example, Schuurs, A.H. et al., *Clinica Chimica Acta*, **81**: 1-40 (1977)), fluorescence (Bauman et al., *Chromosoma*, **84**: 1-18 (1981)), indirect immunofluorescence (Rudkin et al., *Nature*, **265**: 472-473 (1977)), avidin-biotin interactions (Manning, J., et al., *Biochemistry*, **16**: 1365-1370 (1977)), electron microscopy of electron dense nuclei such as ferritin (Broker, T.R. et al., *Nucleic Acids Research* **5**: 363-384 (1978)), latex attachment (Sodja, A., ibid **35**: 385-401 (1978)), combinations of the aforementioned techniques, and others.

The primary recognition event and the signalling event need to be coupled to each other, directly or indirectly, proportionately or inversely proportionately. Thus, in such systems as nucleic acid hybridizations with radiolabeled probes, the amount of radioactivity is usually directly proportional to the amount of analyte present. The same is true with a system such as a sandwich immunoassay, wherein the amount of labeled second antibody being detected is normally directly proportional to the amount of antigen present in the sample. Inversely proportional techniques include, for example, competitive immunoassays, wherein the amount of detected signal decreases the more analyte present in the sample.

The prior art has also utilized amplification techniques, wherein the signalling event is related to the primary recognition event in a ratio greater than 1:1. Thus, the signalling component of the assay may be present in a ratio of 10:1 to each recognition component, thereby providing a 10-fold increase in sensitivity.

The great versatility of polynucleotide sequence-based recognition systems has caused an extensive amount of experimentation and research to be invested therein. This versatility is brought about by the precise alignment of complementary nucleotide bases to each other, adenine (A) aligning to thymidine (T) and guanine aligning with cytidine (C). Given this complementarity, it is possible to utilize any desired sequence to provide an infinitely versatile system.

One of the impediments to a more extensive use of polynucleotide interaction-based systems, however, has been the necessity of attaching signalling or reporter groups (such as radioactive phosphorous, or enzymes, or biotin, or the like) to individual nucleotide

residues in the polymer chain. At least two problems arise out of this requirement.

First, the chemical reaction conditions involved in the modification of a polynucleotide polymer are generally too vigorous to be sufficiently selective for any one nucleotide in particular. For example, dicarbonyl reagents such as kethoxal or glyoxal will indiscriminately react with guanine (see for example Shapiro, R. et al, *Biochemistry*, 5: 2799-2807 (1966), Litt, M., *ibid*, 8: 3249-3253 (1969), or Politz, S.M. et al, *ibid*, 20: 372-378 (1981)). Thus, if one were to use a carbonyl-based cross-linking agent to attach an enzyme or a low molecular signalling compound directly on a polynucleotide chain, one would risk (and in fact one obtains) modification of a substantial amount of all guanine residues in the chain. This, of course, severely hinders the use of such modified chain in a recognition step. This problem has been solved in the prior art by the use of enzymatic (DNA polymerase-based) incorporation of individual modified nucleotides (previously modified in a non-hydrogen-bond-disruptive manner) into a nascent polynucleotide chain. It would, however, be preferred to utilize chemical modification techniques on the final polynucleotide polymer itself.

A second problem is associated with the attachment of signalling groups to polynucleotides and is somewhat related to the first. The problem is based on the necessity of synthesizing, by sometimes sophisticated and elaborate synthetic techniques, the modified monomeric nucleotide units themselves, prior to their enzymatic incorporation into polymer. Thus, radiolabeled nucleotides or biotin-labelled nucleotides have to be independently synthesized. Further, the amount of incorporation of a chemically modified nucleotide into final nucleic acid polymer may also influence the ability of a probe to recognize a given sequence on the analyte. This is particularly important if amplification techniques are utilized wherein signalling groups greatly outnumber recognition groups.

It would therefore be very useful to develop an assay system which utilizes components that are easily prepared, amenable to chemical modification rather than enzymatic-based reactions, that would utilize the great versatility of polynucleotide-based sequence recognition, and include the possibility of signal amplification methods.

The present invention provides a universal assay system which takes advantage of polynucleotide sequence recognitions, which allows for the use of chemical modification reactions, which is also capable of utilizing recognition events based on any type of non-bonding interaction, and which can use any of the myriad of available signalling methods.

The process of the invention comprises a method of detecting in a sample an analyte (A) having a molecularly recognizable portion thereon, which comprises:

forming a complex comprising (1) said analyte bound to (2) a molecular bridging entity comprising a portion capable of recognizing and binding to said molecularly recognizable analyte portion, and a portion comprising a polynucleotide sequence; and (3) a signalling entity comprising a recognition portion capable of complexing to said polynucleotide portion of said bridging entity, and a signal generating portion; and

detecting a signal provided by said signal generating portion present in said complex.

The invention provides, in addition to the aforementioned process, various elements and components to be used therein, such as various molecular bridging entities, and various signalling entities, as well as kits comprising said entities, and other components for use in the process.

In essence, the invention is based on the realization that the recognition portion and the signalling portion of the multi-component assay system should be present on different components of the system, thereby separating them, and avoiding the interference of the signalling portion on the recognition portion. This separation into multi-component entities, also allows the signalling portion to be attached to one of the components by chemical modification techniques, without affecting the recognition component.

Uses for the process, system and components are unlimited, and include all of the uses to which prior art assay techniques have been put, as well as generally, the detection of any analyte capable of recognition, in any sample.

BRIEF DESCRIPTION OF THE FIGURES

The invention will be better understood by reference to the attached Figures wherein:

FIGURE 1 represents a generalized scheme for the assay system of the invention. Analyte 1, having a molecularly recognizable portion 2 thereon, is brought into contact with molecular bridging entity 3, having a portion 4 thereon capable of recognizing the molecularly recognizable portion 2 on analyte 1. Bridging entity 3, in addition, carries a portion 5 comprising a polynucleotide sequence, generally denoted as ATCGATC... . Also present in the system is signalling entity 6 having thereon a polynucleotide portion 7 capable of annealing to polynucleotide portion 5 of the bridging entity 3. The signalling entity 6 also carries a signal generating portion 8. When analyte is present in the sample being analyzed, interaction occurs with bridging entity 3 through the recognizable and recognition portions 2 and 4, respectively. The complex formed thereby is then annealed through the polynucleotide portion 5 to the complementary polynucleotide portion 7 on the signalling entity, which brings the signalling portion 8 into some stoichiomet-

ric relation with the analyt 1.

FIGURE 2 shows a preferr d system und r the broader concept of the invention, wherein analyte 9 comprises a DNA sequence 10 (generally indicat d as ATCGATCGATC). Bridging entity 11, shown as a single-stranded circular polynucleotide polymer, carries a recognizing portion 12 which is a DNA sequence complementary to the DNA sequence of the analyte. Bridging entity 11 also carries, in addition, a poly G sequence 13, which is capable of annealing and forming a stable hybrid with complementary poly C sequence 15 on signalling entity 14. Signalling entity 14 also carries a biotin portion 16 as its signal generating group. Presence of the DNA sequence 10 in the sample being analyzed causes the bridging entity 11 to hybridize thereto, and subsequent annealing of the signalling entity to the thus formed complex attaches the biotin portion, through the network, to the analyte. The biotin portion 16 can then be detected, for example by addition of an avidin/enzyme couple, followed by addition of enzyme substrate, and color detection.

SYSTEM COMPONENTS

The term "analyte" as used in the specification and claims includes any substance or substances either alone or in admixtures, which presence is to be detected and, if necessary, quantitated. The analyte may be a molecule of small or high molecular weight, a molecular complex, or a biological system, such as a virus, a cell, or group of cells. Among the common analytes are proteins, polysaccharides, lipopolysaccharides, protein complexes, nucleic acids or segments thereof, either single- or double-stranded, whole viruses or viral components such as cores or capsids, bacteria of various different types, tissue cells, and the like. Among the most common proteins are the structural proteins, enzymes, immunoglobulins, or fragments thereof. Among the most common nucleic acids are DNA and RNA of various different types, such as tRNA, mRNA, rRNA, and the like. Bacteria, either whole or fragments thereof, such as cell walls or other recognizable portions, include both gram positive and gram negative bacteria. Fungi, algae, and other submicroscopic microorganisms are also included, as well as animal (e.g., mammalian) cells.

The analyte should have a "molecularly recognizable portion" thereon. This phrase denotes any molecular portion of the analyte which is capable of being recognized by a complementary molecular portion on the bridging entity of th system. Molecular recognition, as will be understood by thos of skill in the art, includes the non-covalent binding in three dimensions b twe n complementary portions of two molecules. A molecularly recognizable portion on an analyte may be, for xample, a polynucleotid sequence,

such as RNA or DNA, to b r cogniz d by its comple-
m ntary sequence; an antig n portion, to be recog-
nized by its corresponding monoclonal or polyclonal
antibody; an antibody portion, to be recognized by its
corresponding antigen; a l ctin portion, to be recog-
nized by its sugar; a sugar portion, to be recognized
by its lectin; a hormone portion, to be recognized by
its receptor; a receptor portion, to be recognized by
its hormone; an inhibitor portion, to be recognized by
its enzyme; an enzyme portion, to be recognized by
its inhibitor; a cofactor portion, to be recognized by a
cofactor enzyme binding site; a cofactor enzyme bind-
ing site portion, to recognized by its cofactor; binding
ligand recognized by its substrate and vice versa (i.
. biotin-avidin); or any permutation or combinations
thereof.

Among the most common molecularly recognizable portions are the three-dimensional protein ar-
rangements in antigens of various different sorts, the
cell wall structures present in various cells, or the nu-
cleic acid sequences present in the DNA or RNA of or-
ganisms.

The second component of the system is the "mo-
lecular bridging entity". This entity need only contain
a first portion capable of recognizing the molecularly
recognizable portion on the analyte, and a second
portion which comprises a polynucleotide sequence.
These two portions of the bridging entity may be of
the same type (i.e., both of them polynucleotide s -
quences, albeit different ones) or of a different type
(one being, for example, an antibody portion and the
other the polynucleotide portion.)

The portion on the bridging entity capable of rec-
ognizing the molecularly recognizable portion on the
analyte must contain a molecule or molecular frag-
ment complementary to the recognizable portion on
the analyte. Therefore, if the analyte contains a poly-
nucleotide sequence, the recognizing portion of th
bridging entity should be a complementary polynu-
cleotide sequence or "probe". If the molecularly rec-
ognizable portion on the analyte is a generalized an-
tigen, the recognizing portion on the bridging entity
should be an antibody thereto. The same is true with
respect to the complementary pairs sugar/lectin,
receptor/hormone, inhibitor/enzyme, and the like, de-
scribed previously.

The second portion of the molecular bridging en-
tity must comprise a polynucleotide sequence. The
polynucleotide sequence can be any chosen se-
quence, provided that it is long enough to provide sta-
ble annealing with a complementary sequence under
given stringency conditions, that it be complementary
to the polynucleotide sequence on the signalling en-
tity, and, if th recognizing portion on the bridging en-
tity is its If a polynucleotid sequence, that it b suffi-
ciently different from said recognizing sequenc
portion, to avoid hybrid formation betw n the analyt
sequ nce and the second polynucleotide portion

on the bridging entity. The latter of the three conditions is required to prevent molecular confusion with concomitant appearance of false results.

The second portion polynucleotide sequence on the bridging entity (i.e., the complementary to the sequence on the signalling entity) may code for a particular gene product or products, or may code for no gene product at all. Thus, any structural gene or portion thereof could be used as the polynucleotide sequence portion on the bridging entity. A preferred sequence, however, would not code for a given gene since such coding may interfere with complementary gene sequences present in the analyte. It is thus preferred to choose polynucleotide sequence portions on the bridging entity which are non-coding, and not likely to be complementary to sequences on the analyte such as, for example, sequences comprising poly deoxy G, poly deoxy A, poly deoxy GT, poly deoxy GA, poly deoxy GAT, poly deoxy GTA, or any other low complexity (repeating) sequence. By "polynucleotide" is meant to include both polyribonucleotides, polydeoxyribonucleotides, or any poly-purine, -pyrimidine or analog and combinations thereof.

Specific examples of bridging entities as used in this invention are covalently attached entities of monoclonal or polyclonal antibodies with polynucleotides, polynucleotides with polynucleotides, protein antigens with polynucleotides, saccharides with polynucleotides, small molecular weight organic compounds with polynucleotides, lectins with polynucleotides, receptors with polynucleotides, hormones with polynucleotides, enzyme inhibitors with polynucleotides, enzyme cofactors with polynucleotides, and combinations and permutations thereof.

The molecular ratio of the recognizing portion on the bridging entity, to the polynucleotides sequence portion thereon need not necessarily be 1:1. There may be many more polynucleotide sequence portions than recognizing portions, or vice versa. In the case when the ratio of polynucleotide sequence portion to recognizing portion on the bridging entity is greater than 1, for example, 5, 10 or greater, the system amplifies the primary recognition event by a factor equal to the ratio.

Among preferred bridging entities of the invention are circular polymers of single- or double-stranded DNA. The single-stranded ones include so-called filamentous phages, such as fd, fI and M13 (see Van Wezenbeek, P., Gene, 11: 129 (1980).) These filamentous phages do not lyse their host; rather, they are released from infected cells as the cells continue to grow and divide. M13 is commercially available (Bethesda Research Labs, Inc.) and has been extensively used as a cloning and sequencing system. It can be cut at a restriction endonuclease site to incorporate ther in any desired polynucleotide probe sequence, to serve as the recognizing portion of the bridging entity. Either at the same site or at a different

site, the circular DNA can be opened to incorporate the polynucleotide portion of the bridging entity, capable of annealing to the complementary portion on the signalling entity. In this manner, a bridging entity is obtained which is capable of recognizing a gene sequence on an analyte by hybridization, and is also capable of annealing to the signalling entity through another sequence thereon. (A generalized system of this sort is shown in FIGURE 2.)

In one particularly preferred embodiment, the bridging entity comprises a DNA polymer which carries the sequence for a given gene (for example, a viral probe such as hepatitis B virus, EBV and the like), and, at another place in the polymer, a poly G, or poly GT, or poly dG, or poly dC, or poly dCA, or poly dGdT polynucleotide portion. Ideally, a single-stranded DNA polymer can be provided carrying the polynucleotide portion capable of annealing to the signalling entity (e.g., poly dGT), and also carrying a restriction endonuclease site, so that the user can incorporate any desired DNA probe thereinto. In this manner, by a few simple enzymatic manipulations, the DNA polymer bridging entity can be quickly transformed into a wide variety of bridging entities.

The signalling entity of the invention needs to carry both a polynucleotide portion capable of annealing to the complementary portion on the bridging entity, and a signal generating portion.

The polynucleotide portion on the signalling entity is defined by the same parameters as the complementary portion on the molecular bridging entity. It should be of a length capable of forming stable polynucleotide hybrids with the corresponding polynucleotide on the bridging entity. "Annealing" as used in this part of the invention refers to the required base pair matching between two complementary polynucleotide strands, under any given set of stringency conditions. It is generally understood in the art that about 12 to 13 nucleotides in a row are necessary for stable annealing. Thus, as a minimum, the number of nucleotides in the sequence should be that necessary for stable annealing with the polynucleotide portion of the bridging entity. The formation of the hybrid should be stable enough to withstand any washing, elution, or signal detection procedures which follow hybridization.

The "signal generating" portion of the signalling entity can encompass virtually any of the signal generating systems used in the prior art, and any system to be developed in the future. It comprises a moiety which generates a signal itself (e.g., a radiolabel), or a moiety which, upon further reaction or manipulation will give rise to a signal (e.g., an enzyme-linked system). Both types are herein called "signal generating" portions.

Thus, the signal generating portion may comprise a radiolabel (e.g., ¹⁴C, ³²P, ³H, and the like), an enzyme (e.g., peroxidase, alkaline or acid phosphatase,

tase, and the like), a bacterial lab I, a fluor sc nt label, an antibody (which may be used in a double antibody system), an antigen (to be used with a labeled antibody), a small molecule such as biotin (to be used with an avidin, streptavidin, or antibiotin system), a latex particle (to be used in a buoyancy or latex agglutination system), an electron dense compound such as ferritin (to be used with electron microscopy), or any combinations or permutations thereof.

For example, if the signal generating portion of the signalling entity is an antigen, a signal can be generated by complexing said antigen with an antibody/enzyme conjugate, followed by addition of enzyme substrate. If the signal generating portion of the signalling entity were an antibody, signal can be generated by complexing anti-antibody or an F_c binding protein such as Protein A therewith, which second antibody or Protein A have been conjugated to an enzyme.

Among the preferred signal generating portions are those based on the biotin/avidin system. This system can be incorporated into the signalling entity by a variety of means. For example, the polynucleotide portion of the signalling entity can be covalently attached to biotin via a cytochrome c bridge (Manning et al, Biochemistry, 16: 1364-1370 (1977), Manning et al, Chromosoma, 53: 107-117 (1975), Sodja, A., Nucleic Acids Research, 5: 385-401 (1978)), or the biotin can be covalently incorporated into specific nucleotide residues (Langer, P.R., Proceedings of the National Academy of Sciences, USA, 78: 6633-6637 (1981)), or the biotin can be attached to a polynucleotide by means of a diamine (e.g., pentane diamine) bridge (Broker, T.R., et al, Nucleic Acids Research 5: 363-384 (1978)). Interaction of the biotin molecules in the signal generating portion with avidin, streptavidin or antibiotin antibodies is then carried out, wherein the avidin, streptavidin or the antibodies are conjugated to such signalling components as latex particles (Sodja, A., et al, supra, or Manning, et al Chromosoma, supra), ferritin (Broker, supra), a fluorogen such as fluorescein, an enzyme, or the like,

A thorough description of various non-radioactive signal generating systems, both biotin/avidin-based and non-biotin/avidin-based can be found in two presently copending patent applications: Publication Number 82-301804.9 filed at the European Patent Office on April 6, 1982 to "Modified Nucleotides and Methods of Preparing and Using Same" by Ward et al (EP-A-63879), and Publication Number 83-106112.2, filed on June 22, 1983 at the European Patent Office to "Modified Nucleotides, Methods of Preparing and Utilizing, and Compositions Containing the Same" by Engelhardt et al (EP-A-97373).

In addition, the signal generating portion of the signalling entity need not be a polynucleotide which has been chemically modified or artificially altered in any way. Some biological systems perform *in vivo*

modifications which can be utilized by this system. One such system is the phage T₄ grown in E. coli. T₄ DNA has a very high content of glycosylated C residues. It is possible to insert (clone) a low complexity repeating polynucleotide sequence into phage T₄. This phage would then be naturally propagated and glycosylated in the host. The viral DNA can be isolated from E. coli and bound to a complementary sequence on the bridging moiety. Detection could then be accomplished via a lectin/enzyme system, or lectin/fluorescent dye, or lectin/electron dense material, or lectin/radioactive label, using the natural glucose residues on the T₄ DNA as points of anchorage. Other T (even) phages such as T₂, T₆, or T₈ can also be used.

The number of signal generating portions need not have a 1:1 stoichiometry with the number of polynucleotide portions on the signalling entity. When the ratio of signal generating portions to polynucleotide portion in the signalling portion of the signalling entity is greater than 1 (e.g. greater than 5, or greater than 10), the system functions as an amplification system. Thus, for example, if there are 10 signal generating portions per polynucleotide portion in the signalling entity, a 10:1 signal amplification over the bridging entity is obtained. If, in addition, the bridging entity has a signal amplification system itself, i.e., the ratio of polynucleotide portions to recognizing portions on the bridging entity is greater than 1, the overall signal amplification system is the product of both ratios. This means that for every primary recognition event occurring at the level of analyte, the amplification quickly increases and leads to very sensitive systems. This factor can be readily controlled by the design of the system components.

PROCESSES OF PREPARATION

The bridging entity, as stated previously, needs to comprise a recognizing portion and a polynucleotide portion. The signalling entity requires a polynucleotide portion and a signal generating portion. Thus, generally, the method of preparation of individual components in the system will relate to the covalent attachment of polynucleotides, or individual components thereof, to 1) protein moieties, 2) saccharide moieties, 3) other polynucleotide moieties, 4) small molecular weight compounds (e.g., MW less than about 1000), 5) radiolabels, or 6) insoluble phases such as bacterial particles, or latex particles. Therefore, the chemistry involved in the covalent attachment or conjugation of nucleic acids to their corresponding partner or partners is well within the skill of the art.

The covalent attachment of polynucleotide sequences to proteins is well described in the literature. Normally, the reaction is carried out directly by carbodiimid crosslinking (Halloran, M.K. J. Immunol. 373

(1966) or by cross-linking the protein to the nucleic acid in the presence of such agents as formaldehyde (see e.g., Brutlag, D. et al., *Biochemistry*, 8: 3214-3218 (1969), Manning, J.E., et al., *Chromosoma*, 53: 107-117 (1975)), (4-azidophenyl) glyoxal (Politz, S.M., *Biochemistry*, 20: 372-378 (1981)); by oxidation of 2',3'-hydroxy ends of a polyribonucleotide, followed by i) Schiff base formation with the amine groups of a protein, and by ii) borohydride reduction (Sodja, A., et al., *Nucleic Acids Research*, 5: 385-401 (1978)). Other methods include direct bromination of DNA (Jones, A.S., *Nature* 183: 1603 (1959) followed by reaction with diaminohexane (Lowe, C.R., *Eur. J. Biochem.* 73: 265-274, (1977)), and coupling via protein carboxyl functions; or by mercuration of cytosine moieties (Dale, R.M.K. et al., *P.N.A.S.*, 70: 2236-2242, (1973)) followed by halogenation (Dale, R.M.K. et al., *Nucleic Acids Res.* 2: 915-930 (1975)), reaction with diaminohexane and coupling to protein carboxyl groups.

Of particular interest is the use of dicarbonyl reagents for chemical modification of guanine bases in the preparation of the signalling entity. This represents one of the particularly useful advantages of the present multi-component assay system. If the polynucleotide portion on the signalling entity has very low G content, it is possible to chemically react said polynucleotide portion with any material via a cross-linking agent such as a dicarbonyl compound, without fear of irreversibly modifying the annealing properties of the polynucleotide portion in question. This applies equally well to the attachment of any small molecular weight molecule to the polynucleotide portion, which attachment depends on the use of dicarbonyl compounds, or other nondiscriminating cross-linking agents. This technique saves the effort and time involved in previously modifying individual nucleotide residues, and then incorporating these into a polynucleotide strand by enzymatic polymerization.

The attachment of polynucleotide sequences to saccharides can be carried out according to Cramer et al., *Chem. Ber.* 92: 384-391 (1959). Saccharides having up to 20 saccharide units are preferred.

The attachment of polynucleotide sequences to other polynucleotide sequences is carried out by either chemical or enzymatic techniques, such as using blunt end ligation or ligation based on the presence of cohesive termini generated by endonuclease digestion enzymes. The cleavage and ligation of DNA sequences to each other is well described in Helling and Lomax, "The Molecular Cloning of Genes-General Procedures" which is Chapter 1 of "Genetic Engineering" by Chakrabarty, CRC Press, 1978, pages 1-30.

Other methods for attaching polynucleotides to polynucleotides include using SS Dna + Ribo dUTP + Terminal Transferas (Roychoudery, R. + Wu, R., in *Meth. in Enz.*, LXV, 43, (1980)); Periodate oxidation, reductive amination with amino derivatives including

5 1,6 diamino hexane (1), 3-aminopropionic acid (2), or bis (2-amino ethanethiol) (3), (Perikeh, I Mach, S. and Cuatrecasas, in *Meth. in Enz.* XXXIV, 82 (1974)); or by limited bromination of C (through mercuration) (Dale & Ward supra) and subsequent reaction of DNA with same reagents ((1), (2) + (3)).

10 DNA derivatives of compounds (1) or (2), above, can subsequently be coupled to proteins via water-soluble carbodiimide derivatives (Inman, J.K. in *Meth. in Enz.*, XXXIV, 52-53) (1974). In case (3), the protein can be activated with the N-hydroxy succinimide ester of bromoacetic acid. The resulting activated protein can be covalently linked to the thiolated nucleic acid at room temperature.

15 The covalent incorporation of radiolabels such as ³²P into DNA sequences can be done by any of a variety of methods, such as direct incorporation of radiolabeled nucleotides by enzymatic polymerization, nick translation, and the like (Rigby et al., *J. Mol. Biol.* 113: 237-251 (1977)).

20 The preparation of the individual elements of the signal generating system such as protein/latex conjugates, protein/ferritin conjugates, antibody/enzyme conjugates, fluorogen/antibody conjugates, avidin/enzyme conjugates, and the like is generally well known in the art and will be not described in further detail.

25 The specific preparation of individual polynucleotide sequences is also well understood by those of skill in the art. For example, if a polynucleotide sequence comprises a gene or genes, the same can be prepared by synthetic procedures, or can be prepared by reverse transcription of mRNA using reverse transcriptase to generate a complementary DNA. If the polynucleotide sequence comprises a strand of any one nucleotide (e.g., poly dG or poly dC) or a strand of any dinucleotide pair (e.g., poly dGT, or the like), the same can be readily prepared by enzymatic-based reactions such as by using DNA polymerase, or by synthetic methodology.

METHODS OF USE

30 The analyte being detected can be present in any biological or non-biological sample, such as clinical samples, for example, blood, urine, feces, saliva, pus, semen, serum, other tissues, fermentation broths, culture media, and the like.

35 If necessary, the analyte is preextracted or purified by methods known to concentrate the particular type of analyte from its admixing components. For example, if the analyte is a protein or protein-containing fraction, protein extraction procedures such as salt precipitations, alcohol precipitations or chromatography can be utilized. If the analyte comprises a nucleic acid segment to be identified, nucleic acid extraction procedures, such as phenol extraction, can be utilized. The analyte, together with impurifying

materials if such be the case, can be tested in the mixture as purified or, especially when it is a nucleic acid segment, can be immobilized (see for example, Wahl et al U.S. Patent 4,302,204.)

The composition suspended containing the analyte is incubated with the bridging entity for a time and under conditions sufficient to allow complexation between the recognizable portion of the analyte and the recognizing portion on the bridging entity. These conditions will vary depending on the nature and amount of the analyte and of the bridging entity. Normally, after complexation has occurred, the sample is washed with neutral solution to remove excess bridging entity. Alternatively, no wash is carried out at this stage but signalling entity is added to the mixture and a wash is carried out after annealing has occurred between the polynucleotide strands on the bridging entity and on the signalling entity respectively. Hybridization of the bridging entity strand to the signalling entity strand is carried out under hybridizing conditions and under any set of stringency conditions. A final wash may be necessary prior to generation of signal.

Signal generation is carried out by any given technique, depending on the nature of the signal generating system. Thus, if an enzyme linked assay is utilized, the ternary complex between analyte, bridging entity and signalling entity is allowed to incubate with the enzyme carrying reagent (e.g., enzyme/antibody conjugate), and substrate is added thereto to develop color. Alternatively, enzyme might be attached directly to the polynucleotide strand on the signalling entity, in which case substrate is added immediately thereafter to obtain color development. If the signal generating portion of the signalling entity is a biotin moiety, then a biotin reactive molecule such as avidin, streptavidin or anti-biotin antibody, is added thereto. The biotin reactive molecule is conjugated to an enzyme, a fluorescent compound, an electron dense compound, or an insoluble solid phase, and detection is carried out by appropriate means.

APPLICATIONS

The applications of the system of the invention are unlimited. Any analyte desired to be detected and analyzed in any sample can be subject to the method of the invention.

For example, the system can be used for microorganism detection and identification, by using any of a variety of recognizable portions and recognizing portions in the analyte and the bridging entity respectively.

Of particular interest is the detection and identification of viral and bacterial DNA sequences.

The method can be utilized to diagnose genetic disorders by preparing a polynucleotide complementary to a DNA sequence which is associated with

the genetic disorder, and detecting the presence of any primary recognition events. Among these genetic diseases, for example, can be mentioned thalassemia. The determination of thalassemia can be made (if known genetic defects) by hybridization of oligonucleotides to genomic DNA followed by specific washing procedures or by restriction analysis and Southern, Northern, or Dot blots.

Another use for the system of the invention is in chromosomal karyotyping, which comprises using a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, and then detecting primary recognition events thereon.

Another use includes a method for identifying or locating hormone receptor sites on the surface of cells, which comprises binding a hormone receptor binding compound present in the bridging entity to the receptor site, and then detecting primary recognition events by means of the signalling system of the invention.

Another use comprises the detection of cancer, by detecting in the blood or serum of suspect subjects, the presence of cancer associated antigens such as CEA (carcinoembryonic antigen). Another use includes a method of tumor or cancer cell identification or detection which comprises identifying malignant cells by detecting the absence of normal receptors sites by the technique of the invention.

Another use includes a method of detecting antibodies against certain infectious diseases in animals, by using antigen therefor as a recognizing portion in the molecular bridging entity. Sugar levels or differential glycosylated hemoglobin levels can be detected in diabetes by using a lectin as the recognizing portion on the molecular bridging entity.

Yet another use for the process and system of the invention is in the insolubilization of analytes. Thus, if a sample is suspected of containing an analyte, and one wishes to extract and purify the analyte from the sample, the "signalling entity" is designed so that the signal generating portion comprises or is capable of specifically binding to an insoluble solid phase, such as a natural or synthetic aqueous insoluble resin, a glass, a plastic such as an acrylate or methacrylate, the inside of a test tube wall, or of a well, and the like. The bridging entity is allowed to incubate with the solid phase, thus creating recognition sites (i.e., affinity surfaces) for the analyte, which is then bound thereto.

The present invention lends itself readily to the preparation of kits comprising one or more of the elements necessary to perform the detection and identification process. Thus, a kit may comprise a carrier being compartmentalized to receive in close confinement therein one or more container means or series of container means such as test tubes, vials, flasks, bottles, syringes, or the like. A first of said container means or series of container means may contain th

bridging entity, for recognition of any of a wide variety of analytes. A second container means or series of container means may contain signalling entities. A third container means or series of container means may contain predetermined amounts of analyte, so as to provide the ability to construct a standard curve into which results can be interpolated. Other container means or series of container means may contain the elements necessary to generate the signal, such as enzyme linked conjugates, avidin linked conjugates, ferritin linked conjugates, latex linked conjugates, fluorogen linked conjugates, and the like.

In a preferred embodiment, the kit carrier contains a first container means comprising a bridging system which is DNA carrying a polynucleotide portion of predetermined sequence and a restriction site or cleavage site on the DNA which can be used to incorporate any of a number of gene probes for testing and identifying genetic sequences associated with the analyte. Another container means in this preferred kit would comprise a signalling entity carrying a polynucleotide portion complementary to the polynucleotide portion present in the DNA present in the first container means, and a signal generating portion which may be any of the aforementioned systems. A third container means or series of container means in this preferred kit may comprise a variety of DNA probes complementary to the genetic sequences present on one or more polynucleotide-containing analytes such as viruses, bacteria, cells and the like.

Thus, the user would utilize a cleavage method (such as use of a restriction endonuclease) to open the DNA in the first container, incorporate thereinto any desired DNA probe present in the third container or container series, ligate the polymer and then utilize the bridging entity and the signalling entity to detect and identify the presence of any desired genetic sequence present in the analyte. It should be kept in mind that a single strand cannot be cut with a restriction enzyme unless a linker (which spans the site) is first hybridized to it, thereby creating a double-strand in that location. Normally the gene would be ligated into the RF (double-stranded) so that it could be amplified in a microorganism. Preferred embodiments of the present invention are explained in detail in the following enumeration:

1. A method of detecting in a sample an analyte (A) having a molecularly recognizable portion thereon, which comprises:

providing a molecular bridging entity (B) having thereon:

(i) a portion capable of recognizing said molecularly recognizable portion on said analyte; and

(ii) a portion comprising a polynucleotide sequence; and

(C) a signalling entity having thereon:

(i) a polynucleotide portion capable of anneal-

ing to said polynucleotide portion of said bridging entity, thereby to form a stable polynucleotide hybrid, and

(ii) a signal generating portion;

forming a complex comprising:

(1) said analyte (A) complexed through said molecularly recognizable portion to

(2) said recognizing portion of said entity (B); said entity (B) being complexed through said polynucleotide portion thereon to

(3) said polynucleotide portion of said signalling entity (C), with the proviso that when said analyte is a polynucleotide, the signal generating portion is not an enzyme that is covalently linked to said recognition portion and

detecting a signal by means of said signal generating portion present in said complex.

2. The method of item 1 wherein said analyte is present in a biological or non-biological sample.

3. The method of item 1 wherein said molecularly recognizable portion on said analyte is proteinaceous.

4. The method of item 1 wherein the molecularly recognizable portion on said analyte comprises nucleic acid.

5. The method of item 1 wherein the molecularly recognizable portion on said analyte comprises a saccharide.

6. The method of any of items 3, 4 or 5 wherein said analyte is selected from the group consisting of an antigen, an antibody, a receptor, a virus, a viral component, a bacterium, a bacterial component, a cell, a cellular component, or any pathogenic or non-pathogenic component of a sample.

7. The method of item 1 wherein said recognizing portion on said bridging entity comprises a polynucleotide sequence.

8. The method of item 1 wherein said recognizing portion on said bridging entity comprises an antigen.

9. The method of item 1 wherein said recognizing portion on said bridging entity comprises an antibody.

10. The method of item 1 wherein said recognizing portion on said bridging entity comprises a saccharide.

11. The method of item 1 wherein said recognizing portion on said bridging entity comprises a lectin.

12. The method of item 1 wherein said recognizing portion on said bridging entity comprises a hormone.

13. The method of item 1 wherein said recognizing portion on said bridging entity comprises a receptor.

14. The method of item 1 wherein said recognizing portion on said bridging entity comprises an enzyme inhibitor or enzyme cofactor.

15. The method of item 1 wherein said recognizing portion on said bridging entity comprises an enzym active site, a c factor binding site, or a receptor protein.

16. The method of item 1 wherein said polynucleotide sequence on said bridging entity codes for a gene product or fragment thereof. 5

17. The method of item 1 wherein said polynucleotide sequence on said bridging entity does not code for a gene sequence or fragment thereof. 10

18. The method of item 1 wherein said polynucleotide sequence on said bridging entity comprises a poly deoxy G, poly deoxy C, poly deoxy T or poly deoxy A sequence, or any poly-ribo or -deoxyribo purine, pyrimidine or analog. 15

19. The method of item 1 wherein said polynucleotide sequence on said bridging entity comprises a sequence portion which is rich in guanosine residues. 20

20. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to another polynucleotide sequence. 25

21. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to an antibody. 25

22. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to an antigen. 30

23. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to a saccharide. 30

24. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to a lectin. 35

25. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to a hormone. 35

26. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to a receptor. 40

27. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to an enzyme inhibitor or enzyme cofactor. 45

28. The method of item 1 wherein said polynucleotide sequence in said bridging entity is covalently attached to an enzyme. 45

29. The method of item 7 wherein said bridging entity is a circular DNA polymer. 50

30. The method of item 29 wherein said DNA is single-stranded. 50

31. The method of item 29 wherein said circular DNA polymer is derived from a filamentous phage. 55

32. The method of item 31 wherein said filamentous phage is M13 or a variant thereof.

33. The method of item 32 wherein said M13 phage carries a sequence portion which is rich in guanosine residues, or cytosine residues.

34. The method of item 1 wherein said polynucleotide portion on said signalling entity codes for a gene product or fragment thereof.

35. The method of item 1 wherein said polynucleotide portion on said signalling entity does not code for a gene product or fragment thereof.

36. The method of item 1 wherein said polynucleotide portion on said signalling entity comprises a poly deoxy C, poly deoxy G, poly deoxy A, poly deoxy T sequence, or a repeating sequence of low complexity.

37. The method of item 1 wherein said polynucleotide portion on said signalling entity comprises a sequence portion which is rich in cytosine residues, or guanosine residues.

38. The method of item 1 wherein said signalling entity is a polynucleotide polymer.

39. The method of item 38 wherein said polynucleotide polymer is a naturally occurring modified DNA.

40. The method of item 39, wherein said polynucleotide polymer is derived from a T (even) phage.

41. The method of item 40 wherein said T (even) phage is T₄.

42. The method of item 39 wherein said modified DNA carries a cloned insert.

43. The method of item 38 wherein said polymer is single-stranded.

44. The method of item 43, wherein said polymer is derived from a filamentous phage.

45. The method of item 44 wherein said phage is M13 or a variant thereof.

46. The method of item 1 wherein said signal generating portion of said signalling entity is radiolabeled.

47. The method of item 1 wherein said signal generating portion of said signalling entity is not radiolabeled.

48. The method of item 47 wherein said signal generating portion comprises an enzyme.

49. The method of item 47 wherein said signal generating portion comprises a biotin moiety.

50. The method of item 47 wherein said signal generating portion comprises a fluorogenic compound.

51. The method of item 47 wherein said signal generating portion comprises an electron dense compound.

52. The method of item 47 wherein said signal generating portion comprises or binds to an insoluble phase.

53. The method of item 52 wherein said insoluble phase comprises a latex particle, a resin, or a bacterium.

54. The method of item 47 wherein said signal

g neration portion comprises an antibody or antigen.

55. The method of item 47 wherein said signal generating portion comprises a saccharide or lectin.

56. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises a radioactivity measurement.

57. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises an enzymatic reaction.

58. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises a fluorescence measurement, or electron microscopic measurement.

59. The method of item 47 wherein said signal generating portion is a polynucleotide sequence capable of recognizing a signal containing moiety.

60. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises an antibody/antigen complexation reaction.

61. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises a complexation reaction between biotin and a biotin binding moiety.

62. The method of item 61 wherein said moiety is avidin, streptavidin or an anti-biotin antibody.

63. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises detection of an electron dense compound.

64. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises a complexation reaction between a saccharide and a lectin.

65. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises a binding step on an insoluble phase.

66. The method of item 1 wherein said step of detecting a signal by means of said signal generating portion comprises complexation between a signalling entity comprising a cloned insert on a naturally-occurring modified DNA, and the bridging moiety, followed by binding a modified lectin to said signalling entity.

67. The method of item 66 wherein said modified DNA is derived from a T₄ phage.

68. The method of item 65 wherein said insoluble phase is a latex particle.

69. The method of item 1 wherein said recognizable portion on said analyte is a polynucleotide sequence, said recognizing portion on said bridging entity is a polynucleotide sequence capable of stably annealing thereto, said bridging entity is

5 a single-stranded DNA polymer, and said step of detection by means of said signal generating portion on said signalling entity is based on non-radioactive detection.

10 70. The method of item 69 wherein said bridging entity is derived from a filamentous phage.

15 71. The method of item 69 wherein said signalling entity is derived from a filamentous phage.

16 72. A polynucleotide sequence covalently attached to an antibody.

17 73. The sequence of item 72 wherein said antibody is monoclonal.

18 74. A polynucleotide sequence covalently attached to a lectin.

19 75. A polynucleotide sequence covalently attached to a saccharide having up to 20 saccharide units.

20 76. A polynucleotide sequence covalently attached to receptor.

21 77. A polynucleotide sequence covalently attached to a hormone.

25 78. A DNA molecule carrying a polynucleotide portion which comprises a sequence selected from the group consisting of poly dGT, poly dAC, poly dCT, poly dAT, poly dGC, poly dGA, poly dG, poly dC, poly dT, poly dA, and a repeating low-complexity polynucleotide.

30 79. The DNA molecule of item 78 which is a filamentous phage.

80. The phage of item 79 which is M13 or a variant thereof.

81. The DNA molecule of any of items 78 or 79 wherein said sequence is at least an oligonucleotide.

35 82. The DNA molecule of any of items 78 or 79 which also carries a polynucleotide sequence complementary to part or whole of a gene sequence of a nucleic acid-containing organism.

40 83. The DNA molecule of item 82 wherein said organism is a virus, a prokaryotic or a eukaryotic cell.

45 84. The DNA molecule of item 83 wherein said prokaryotic cell is a bacterium.

85. The DNA molecule of item 83 wherein said eukaryotic cell is a mammalian cell.

86. The DNA molecule of item 82 which is a filamentous phage.

87. The DNA molecule of item 82 which is M13 or a variant thereof.

88. A circular DNA molecule covalently attached to a non radiolabelled signal generating moiety.

89. The DNA molecule of item 88 which is a filamentous phage.

50 90. The DNA molecule of any of items 88 or 89 which carries a polynucleotide portion which comprises a sequence selected from the group consisting of poly dGT, poly dAC, poly dCT, poly dAT, poly dGC, poly dGA, poly dG, poly dC, poly

dT, poly dA and a repeating low-complexity poly nucleotid .

91. The DNA molecule of any of items 88 or 89 which carries a polynucleotide portion which is rich in cytosine residues.

92. The DNA molecule of item 90 wherein said sequence is an oligonucleotide.

93. The DNA molecule of any of items 88 or 89 which carries a polynucleotide portion which comprises a sequence coding for part or whole of a gene.

94. The DNA molecule of any of items 88 or 89 wherein said signal generating moiety comprises a radiolabel.

95. The DNA molecule of any of items 88 or 89 wherein said signal generating moiety is non-radiolabeled.

96. The DNA molecule of item 93 wherein said signal generating moiety comprises an enzyme.

97. The DNA molecule of item 93 wherein said signal generating moiety comprises a biotin moiety.

98. The DNA molecule of item 93 wherein said signal generating moiety comprises an antibody.

99. The DNA molecule of item 93 wherein said signal generating moiety comprises a fluorogenic compound.

100. A kit useful for the detection of an analyte (A) having a molecularly recognizable portion thereon, comprising:

- I) a carrier being compartmentalized to receive in close confinement therein one or more container means;
- II) a first container means containing a molecular bridging entity (B) having thereon:
 - (i) a portion capable of recognizing said molecularly recognizable portion on said analyte (A); and
 - (ii) a portion comprising a polynucleotide sequence; and
- III) a second container means containing a signalling entity (C) having thereon:
 - (i) a polynucleotide portion capable of annealing to said polynucleotide portion of said bridging entity (B) thereby to form a stable polynucleotide hybrid; and
 - (ii) a signal generating portion.

101. The kit of item 100 which also comprises

- IV) a third container means containing components needed to detect a signal from said signal generating means.

102. The kit of item 100 wherein said recognizing portion on said bridging entity comprises a polynucleotide sequence.

103. The kit of item 100 wherein said recognizing portion on said bridging entity comprises an antigen.

104. The kit of item 100 wherein said recognizing portion on said bridging entity comprises an antibody.

105. The kit of item 100 wherein said recognizing portion on said bridging entity comprises a saccharide.

106. The kit of item 100 wherein said recognizing portion on said bridging entity comprises a lectin.

107. The kit of item 100 wherein said recognizing portion on said bridging entity comprises a hormone.

108. The kit of item 100 wherein said recognizing portion on said bridging entity comprises a receptor.

109. The kit of item 100 wherein said recognizing portion on said bridging entity comprises an enzyme inhibitor or enzyme cofactor.

110. The kit of item 100 wherein said recognizing portion on said bridging entity comprises an enzyme active site or cofactor binding site.

111. The kit of item 100 wherein wherein said polynucleotide sequence on said bridging entity codes for a gene product or fragment thereof.

112. The kit of item 100 wherein said polynucleotide sequence on said bridging entity does not code for a gene product or fragment thereof.

113. The kit of item 100 wherein said polynucleotide sequence on said bridging entity comprises a poly dG, poly dC, poly dT, poly dA sequence; or a low complexity (repeating) polynucleotide.

114. The kit of item 100 wherein said polynucleotide sequence on said bridging entity comprises a sequence portion which is rich in guanosine residues.

115. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to another polynucleotide sequence.

116. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to an antibody.

117. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to an antigen.

118. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to a saccharide.

119. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to a lectin.

120. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to a hormone.

121. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to a receptor.

122. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to an enzyme inhibitor or enzyme cofactor.

123. The kit of item 100 wherein said polynucleotide sequence in said bridging entity is covalently attached to an enzyme. 5

124. The kit of item 100 wherein said bridging entity is a circular DNA polymer. 10

125. The kit of item 124 wherein said circular DNA is single-stranded. 15

126. The kit of item 125 wherein said circular DNA polymer is derived from a filamentous phage. 20

127. The kit of item 124 wherein said filamentous phage is M13 or a variant thereof. 25

128. The kit of item 125 wherein said M13 phage carries a sequence portion which is rich in guanosine or cytosine residues. 30

129. The kit of item 100 wherein said polynucleotide portion on said signalling entity codes for a gene product or fragment thereof. 35

130. The kit of item 100 wherein said polynucleotide portion on said signalling entity does not code for a gene product or fragment thereof. 40

131. The kit of item 100 wherein said polynucleotide portion on said signalling entity comprises a poly dC, poly dG, poly dA, poly dT sequence, or a low-complexity, repeating polynucleotide. 45

132. The kit of item 100 wherein said polynucleotide portion on said signalling entity comprises a sequence portion which is rich in cytosine or guanosine residues. 50

133. The kit of item 100 wherein said signalling entity is a circular DNA polymer. 55

134. The kit of item 133 wherein said DNA is single-stranded.

135. The kit of item 134 wherein said DNA is derived from a filamentous phage.

136. The kit of item 135 wherein said phage is M13 or a variant thereof.

137. The kit of item 100 wherein said signal generating portion on said signalling entity is radiolabeled.

138. The kit of item 100 wherein said signal generating portion of said signalling entity is not radiolabeled.

139. The kit of item 138 wherein said signal generating portion comprises an enzyme.

140. The kit of item 138 wherein said signal generating portion comprises a biotin moiety.

141. The kit of item 138 wherein said signal generating portion comprises a fluorogen.

142. The kit of item 138 wherein said signal generating portion comprises an electron dense compound.

143. The kit of item 138 wherein said signal generating portion comprises or binds to an insoluble phase.

144. The kit of item 138 wherein said insoluble phase comprises a latex particle, a resin, or a bacterium.

145. The kit of item 138 wherein said signal generating portion comprises an antibody.

146. The kit of item 138 wherein said signal generating portion comprises a saccharide.

147. The kit of item 100 wherein said recognizable portion on said analyte is a polynucleotide sequence, said recognizing portion on said bridging entity is a polynucleotide sequence capable of stably annealing thereto, said bridging entity is a single-stranded DNA polymer, and said signal generating portion on said signalling entity is based on non-radioactive detection.

148. The kit of item 147 wherein said bridging entity is derived from a filamentous phage.

149. The kit of item 147 wherein said signalling entity is derived from a filamentous phage. Especially preferred embodiments of the invention are the following:

150. A method of detecting in a sample an analyte (A) having a molecularly recognizable portion thereon, which comprises:

- providing a molecular bridging entity (B) having thereon:
- (i) a portion capable of recognizing said molecularly recognizable portion on said analyte (A), and
- (ii) a portion comprising a recognizable modifier, and

a signalling entity (C) having thereon:

- (i) a receptor for said recognizable modifier capable of attachment or binding to said recognizable modifier of said bridging entity (B) thereby to form a stable complex, and
- (ii) a signal generating portion, and

forming a complex comprising:

- (1) said analyte (A) attached through said molecularly recognizable portion thereon to
- (2) said portion of said molecular bridging entity (B) capable of recognizing said molecularly recognizable portion of analyte (A), said molecular bridging entity (B) being coupled via said recognizable modifier thereon to
- (3) said signalling entity (C) via said receptor thereon capable of attachment to or binding to said recognizable modifier said bridging entity (B).

151. A stable detectable complex comprising a target analyte, a modified first receptor attached thereto via said receptor portion, the modifying portion thereof being attached to a second receptor for said modifying portion of said first receptor, said second receptor having attached thereto a signalling entity.

152. An entity capable of detecting a protein receptor comprising:

- (a) a protein capable of binding said protein receptor, and
- (b) a polynucleotide attached to said protein that is capable of binding said protein receptor.

tor.

153. The entity of it in 152 which further comprises a signal generating portion attached to said polynucleotide.

Having now generally described this invention, the same will be illustrated by reference to certain specific embodiments which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Examples

Examples 1-31 relate to processes of preparation of bridging and signalling moieties. The examples show preparation methods which may be classified within the following categories:

- 1) Chemical activation of oligonucleotides for subsequent coupling to proteins, saccharides and small molecules.
- 2) Chemical activation of proteins for subsequent coupling to DNA, saccharides and small molecules.
- 3) Chemical activation of saccharides for subsequent coupling to DNA, protein and small molecules.
- 4) Chemical activation of small molecules for subsequent coupling to DNA and protein.
- 5) Coupling of DNA to protein, saccharides and small molecules.

The examples classified within the above categories are as follows:

- 1) Chemical activation of Oligonucleotides.
 - A. By terminal ribonucleotide labelling followed by periodic oxidation and reductive amination: Examples 11, 12;
 - B. By non-specific bromination: Examples 28, 29, 30.
 - C. By specific activation of cytosine moieties via 5-iodocytosine: Examples 16, 17 18.
 - D. By specific activation of guanosine moieties via reaction with 3, 4, 5-trichlorodiazobenzene: Example 1.
 - E. By specific activation of adenosine and guanosine-moieties via reaction with 2,3-dibromopropanal: Example 9.
- 2) Chemical Activation of proteins
 - A. By bromoacetylation: Examples 13, 14.
- 3) Activation of saccharides
 - A. By activation of reducing saccharides: Examples 4, 5, 6.
 - B. By activation of non-reducing saccharides: Examples 7, 8.
- 4) Activation of small moieties
 - A. Biotin: Examples 2, 33, 23, 24, 25, 26
 - B. DCTA: Example 3.
- 5) Coupling of DNA to protein, saccharides and small molecules
 - A. To protein: Examples 15, 19, 20.

B. Saccharides: Examples 19, 18.

C. To small molecules: Examples 19, 10, 21, 27.

5 Example 1

Activation of DNA with 3,4,5-Trichloraniline

100 mg of 3,4,5-trichloroaniline were dissolved in 2.5 ml of 0.5M HCl in 50% DMSO and cooled on ice, under vigorous stirring, an equimolar amount of NaNO₂ from a cold 1M solution were added, as rapidly as possible, and then stirring was continued for 10 minutes. 1 mg of Fd DNA in 300 µl of water were mixed with 300 µl of 2M cacodylate buffer pH 6.6 and 500 µl DMSO. (By addition of DMSO the pH of the solution rises to 8.3). 20 µl of the freshly prepared diazonium solution were added thereto and the mixture was incubated for two hours at room temperature. The slight precipitate which appeared during the incubation was removed by centrifugation. The solution was then made 0.4 M with ammonium acetate and the DNA was precipitated with ethanol.

25 Example 1a

Reaction of Trichloroaniline-activated DNA with thiols. Example of Reaction with DCTA-SH and thiol activated mannose

Fd DNA activated with 3,4,5-Trichloroaniline (Example 1) was dissolved in 0.1M sodium hydroxide with an equal amount of 0.1M K₂HPo₄. This solution was treated with an equal volume of 0.1M DCTA-SH (Example 3) or thiol activated mannose (Ex. 6 and 9), and incubated under argon at 65° for 2 hours. The precipitated disulfides were removed by centrifugation and the DNA was purified by G50 chromatography and stored at -20°C. Using radioactive Ni to level the derivatized DNA, it was determined that 60% of guanines had been labelled.

Example 2

Biotin-SH

Three milimoles of Biotin -NHS ester were dissolved in 25 ml of anhydrous DMF and mixed with a 1M solution of cysteamine hydrochloride in 12 ml of 0.5M sodium bicarbonate and the mixture was incubated at room temperature overnight. During the incubation a heavy precipitate appeared. The liquid was removed under reduced pressure at 45°C and the residue was suspended in 50 ml absolute ethanol, 1 g of NaBH₄ was added and the suspension was stirred for one hour at 75°C. The ethanol was removed and cold 1 M HCl was added to bring the pH to 4.5, and the water was removed under reduced pressure at

35°C. (All these operations were performed under an argon atm sphere. to prevent oxidation of the thiol.) The solid residue was powdered and triturated with 4 ml of cold deareated 0.01 M acetic acid. This procedure was repeated twice and the residue was lyophilized. TLC chromatography showed that the main biotin spot contained thiol; two minor spots were thiol negative. In all reactions the amount of biotin used was based on the thiol content.

Example 3

DCTA-SH

One millimole of DCTA-bromide, 1,2-diaminocyclohexane-N, N,N,N-tetraacetic acid, was added to 5 ml of 50% DMF containing 0.2 ml of 2,3-dithioethylene and 0.5 ml of triethylamine. The mixture was incubated under argon for 2 hours at 60-70°C. The solution was then mixed with 20 ml of water and loaded onto a Dowex AG-1 column of 9 ml bed volume. The column was washed with 50 ml of 0.1M acetic acid solution until the flowthrough was thiol free. The DCTA-SH was then eluted with 0.25M HCl. The thiol containing fractions were combined, evaporated to dryness under reduced pressure at 40°C and the free acid (300mg) was stored at -20°C under argon.

Example 4

1-O-Methyl-6-O-Tosyl- α -D-mannopyranoside

Non-reducing saccharides were activated through the primary alcohol group by forming the tosylate, and displacing it with ammonia to form an amino group, or with a dithiol to form a thiol group. An example is described here, the activation of α -methyl-D-mannoside, a non-reducing sugar and of mannose as a reducing one. The tosylation was performed analogous to a published procedure (F. Cramer et al. Chem. Ber. 92, 384-391 (1979).)

23 g of methyl-D-mannoside were dissolved in 400 ml of absolute pyridine and the solution was cooled to -15°C on an ice salt mixture. A solution of 24.6 g of p-Toluene sulfonyl chloride in 80 ml absolute pyridine was added to the vigorously stirred mixture and reacted at -15°C for 30 minutes and 12 hours at 20°C. The pyridine was removed under reduced pressure at 40°C and the residue was dissolved in chloroform. The solution was warmed to 50°C and washed successively with 0.5 M potassium hydrogen sulfate followed by 0.5M potassium bicarbonate solution at 50°C (the 50°C green temperature was necessary to avoid gel formation).

Example 5

6-amino- α -D-mannoside hydrochloride

5 As solution of 6 g of the tosylate (Example 4) in 130 ml absolute methanol was saturated at 1°C with dry ammonia and autoclaved for 16 hours at 120°C. The dark reaction product was refluxed with charcoal and methanol was removed by distillation leaving a slight yellow syrup. The syrup was dissolved in water and the sulfonate liberated during the displacement reaction was removed by passing the solution through an anion exchanger. HCl was added to the elutant to bring the pH to 5.0 and the water was removed under reduced pressure at 40°C. The residue was triturated with a mixture of 15 ml of absolute methanol and 15 ml of absolute ether and the solid material was dissolved in 50 ml of absolute methanol and cooled, the addition of 25 ml absolute ether initiated crystallization, yielding 2.5 g hydrochloride.

Example 6

S-(2-mercaptoethyl)-6-thio- α -D-methylmannopyranoside

6 g of the sugar tosylate (Example 4) were dissolved in 250 ml of absolute methanol containing 20 ml of a freshly prepared solution of sodium methoxide. To the mixture was added 5 ml of 1.2 ethanethiol. The mixture was autoclaved at 120°C for 10 hour and the reaction product was treated as above. Yield 3.1 g.

Example 7

2,3,4,6-Tetraacetyl- α -D-mannopyranosyl chloride

This compound was prepared analogously to a published procedure (D. Horton, Organic Synthesis Vol 46 p. 1, Wiley N.Y. 1966). 25 g of dried mannose were added slowly with stirring to 60 ml of acetyl chloride. The vessel was connected to a reflux condenser and the mixture was stirred for 16 hours at room temperature. Chloroform, 300 ml, was added through the condenser and the mixture was poured with vigorous stirring onto 300 g of ice and 100 ml of water. The mixture was transferred to a separatory funnel, and the organic phase was poured as fast as possible into a beaker containing ice and 300 ml of saturated sodium bicarbonate solution. The organic phase was separated and dried with 25 g of anhydrous magnesium sulfate. The drying agent was removed, washed with dry alcohol free chloroform and the combined chloroform solution was concentrated to 35 ml at a reduced pressure in a rotatory vaporator. At 50°C ether was added to the solution until slightly turbid and the solution left at room temper-

ture. The crystals were removed by filtration and washed with dry ether. Yield 39 g.

Example 8

S-(2-mercaptoproethyl)-1-β-D-mannopyranosyl sulfide and S-(2-aminoethyl)-1-β-D-mannopyranosylsulfide

To a solution of 10 g acetochloromannose (Example 7) in 60 ml anhydrous DMF, 4 ml of 1.2 ethanedithiol or 5 g cysteamine hydrochloride and 5 g of fine powdered sodium carbonate were added to the suspension which was stirred under argon for 6 hours at 70°C. The carbonate was removed and the liquid was evaporated under reduced pressure at 45°C. The residue was dissolved in absolute methanol and freshly prepared 0.1 M sodium methoxide was added to bring the pH to 8.0 and the mixture was stirred for 5 hours at room temperature. 2 ml of glacial acetic acid were added and the liquid was removed under reduced pressure. The residue was recrystallized from acetic acid. Yield 3.1 g.

Example 9

Activation of DNA with 1,2 dibromopropanal

A solution of acrolein (1.7 g) in ether was cooled on an ice bath and 1.3 ml of bromine were slowly added under stirring while waiting for the color to disappear for the next bromine addition. The ether was partially removed by blowing argon over the solution, resulting in a 2 M solution of the 1,2-dibromopropanal (Example 8). The DNA used for the following operations was in the triethylammonium form to facilitate solution in DMF.

0.5 mg of 3H fd-DNA (linear) (partly tritiated) in 250 µl of water were mixed with 3.0 ml of 0.5 M triethylammonium acetate, pH 4.5 in 70% ethanol and 50 µl of the dibromopropanal solution was added. The mixture was stirred in the dark at 37°C for 40 hours. The reaction was monitored by the appearance of fluorescence. The reaction mixture was evaporated to dryness under reduced pressure, and the DNA dissolved in 0.6 ml water and desalting by G 50 filtration with water as the eluent. The fractions containing radioactivity were combined and the volume was reduced to 0.2 ml.

Example 10

Labelling of the 3,4,5-trichloroaniline DNA with DCTA-SH

0.5 mg of the activated DNA (Example 1) in 0.2 ml of water were mixed with 2.0 ml of 0.5 M triethylammonium acetate in 90% DMF and 50 mg of DCTA-SH in the triethylammonium form were added. The

mixture was stirred in the dark for 4 hours at 50°C. The DMF was removed under reduced pressure at 45°C and the DNA was desalting by G-50 filtration. The degree of labeling was then determined by the use of radioactive Ni63. On the average every 5.3 bases were labelled, by calculation.

Similar procedures were used for the biotination and glycosylation of the activated DNA using the thio derivatives of these substances (Examples 2, 6 and 8).

Chemical labelling of DNA

Rationale

Guanosine couples with certain diazonium salts at the 8 position to give stable colored products (H. Fischer, Z. Physiol. Chem. 60, 696-78, (1909)), and at position 2 to form yellow products which are acid labile (H. Kossel, Z. Physiol. Chem. 340, 210, 1965, E.N. Moudrianakis et al., Biochim. Biophys. Acta 123, 421 (1966)). The guanosine residues in the single stranded nucleic acids can couple with diazonium salts and this reaction has been used to fix single stranded nucleic acids to cellulose (J.C. Alwine et al. Methods in Enzymology Vol. 68, p. 220-242, 1979)). If the coupled diazonium compound contains an active group that can be easily substituted by thiols or amines, then this constitutes an easy method to attach biotin or other groups to single stranded nucleic acids. 3,4,5-trichlorophenyl diazonium chloride is such a substance which has been used by the inventors to add biotin, 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid (DCTA), and some sugars to a single stranded DNA.

Another possibility to label single stranded nucleic acids is based on the fact that chloracetaldehyde reacts with adenine at pH 4.5 to form a fluorescent etheno derivative (under mild conditions). J.R. Barrio, et al Biochem. Biophys. Res. Commun. 46, 597-604, 1972), cytidine reacts at pH 3.5 and guanine at pH 6.5. At pH 4.5 guanine does not react at all (P.D. Sattsangi, et al, J. Org. Chem., 42, 3292-3296, (1977).)

By using 1,2 dibromopropanal in place of chloracetaldehyde it was possible to derivatize DNA with an active primary bromide group, which, under mild reaction conditions, reacts with thiol or amine derivatives, offering another method of labeling DNA. These two methods are base specific.

Example 11

Terminal addition of 5'-uridine monophosphate (UMP) to linear 3H fd-DNA with Terminal Transferase and 5'-uridine triphosphate.

The incubation mixture of 600 µl contained: 400 µg of DNA, 1mM CoCl₂, 0.2 mM dithiothreitol, 0.1 M

cacodylic acid, 25 mM tris bas , 1 mM UTP and 400 units of terminal transferas . Th final pH of the mixture was 6.9-7.0.

The mixture was incubated for 2 hours at 35°C. The DNA was precipitated with ethanol and dissolved in 400 µl 0.2 M sodium acetate pH 4.7.

Example 12

Oxidation of the Terminal Ribo Group and Reductive Amination. Synthesis of Amino, carboxy and thio-end substituted DNA.

The incubation mixture of 450 µl contained: 400 µg terminal labeled DNA (Example 11), 0.2 M sodium acetate pH 4.7 and 0.1 M NaIO₄. It was incubated for two hours at room temperature in the dark and the mixture was passed through a G 50 column equilibrated in 0.3 M potassium borate pH 9.0-9.3, fractions of 0.2 ml were collected. All radioactive fractions were combined in a total volume 1.2 ml. The DNA solution was made 0.4 M with one of the amino components (ϵ -aminocaproic acid, cysteamine, or 1,6-diaminohexane, using a stock solution of 1 M which was adjusted to pH 9.3), and was incubated in the dark for 90 minutes at room temperature.

The resulting Schiff base was reduced with NaBH₄ as follows: NaBH₄ freshly dissolved to 0.2 M in water (1 ml) to M was added in four portions over 30 minute intervals. The incubation was continued for a total of 3 hours. The salts and the excess of the amino component were removed by G 50 filtration in a column equilibrated in 0.4 M sodium acetate containing 1mM beta-mercaptoethanol, the DNA-containing fractions were then combined and stored over argon at -70°C. Before use, DNA was precipitated with ethanol and dissolved in the desired buffer.

Example 13

Activation of bromoacetic acid N-hydroxysuccinimide ester

The NHS (N-hydroxy succinimide) ester of bromoacetic acid was prepared as follows: 100 mmoles of (13.9 g) of bromoacetic acid were dissolved in 50 ml of anhydrous DMF, to this solution 100 mmoles (20.6 g) of N-N-Dicyclohexylcarbodiimide were added with stirring followed by 100 mmoles of N-hydroxysuccinimide (11.9 g adjusted for 100% purity), and the mixture was stirred for 6 hours at 37°C. The mixture was then placed for 2 hours at -20°C to accelerate the precipitation of hydroxyurea, which was removed by filtration. In the filtrate the DMF was removed under reduced pressure at 45°C and the active ester was recrystallized from 2-propanol.

Example 14

Brom acetylation of IgG

5 IgG (20 mg/ml) in 0.3M potassium borate buffer pH 9.9 was mixed with 0.06 vol of a 10 mg/ml solution of the NHS ester of bromoacetic acid (Example 13) in DMSO, and the mixture was gently mixed for 1 hour at room temperature. The sample was then dialyzed against 0.1 M NaCl 0.1M phosphate buffer pH 7.5.

Example 15

Synthesis of DNA-IgG Conjugates

15 1.6 mg/ml of bromoacetylated IgG (Example 14) in 0.3 M potassium borate buffer were incubated at room temperature under argon with a thio-substituted, end labeled DNA solution (Example 12) of 3 mg/ml for 2 hours. Mercaptoethanol 0.01 M was then added and the mixture was further incubated at the same temperature for 2 hours to quench the unreacted bromine residues. The solution was then adsorbed on a Protein A column, and the unconjugated DNA was eluted with 1.0 M NaCl. The DNA-IgG conjugate and the unreacted IgG were eluted with isothiocyanate, and dialyzed against 0.1M phosphate buffer, pH 7.2 to remove isothiocyanate. The conjugated IgG and the free IgG were then precipitated with ammonium sulfate 50%, the pellet dissolved in 1.0 ml phosphate buffer and the free IgG was separated from the conjugated IgG by fractionation on a Bio-Gel p-300 column equilibrated in .01M NaCl, 0.1M phosphate pH 7.2.

Example 16

Mercuration of pBR322

40 pBR322 DNA (100 µg) dissolved in 1 ml of 5mM sodium acetate pH 7.5 containing mercuric acetate (3 mg, .01 mmol) was reacted for 4 hours at 50°C according to a procedure of Dale et al (Nucl. Acid Res. 2:915, 1975). The 5-cytosine mercurated DNA was exhaustively dialyzed in 0.01 M Tris HCl, pH 7.5, containing 0.02 sodium chloride 2mM EDTA.

Example 17

Iodination of Mercurated pBR 322 DNA

55 To the mercurated DNA from the previous experiment in 1 ml 0.1M Tris HCl pH 7.5 was added 1 mg iodine using the procedure of Dale et al (Nucl. Acid Res. 2, 915, 1975). After reaction at 20°C for 2 hours the excess I₂ was extracted with chloroform and the iodinated DNA was dialyzed against 0.01m Tris HCl pH 7.5 containing 0.02M sodium chloride and 2mM

EDTA. The substituted DNA was analyzed by successive digestion with *N. crassa* endonucleases, snake venom phosphodiesterase DNase, and *E. coli* alkaline phosphatase (H. Yamasaki et al., Cancer Res. 37:1389, 1977). The mixture was eluted through DE-52 amino cellulose, and the nucleotides were analyzed by reverse phase HPLC using authentic 5-iodo-2'-deoxyuridine as a standard.

Example 18

Reaction of 5-Iodocytosine pBR322 DNA with amines.

Example of reaction with 1,6-Diaminohexane.

To the iodinated DNA from the previous reaction dialyzed into 1 ml 0.1 M sodium borate was added 116 mg (1 mmol) diaminohexane. The reaction mixture was flushed with argon and heated at 100° for 2 hours. The aminohexyl substituted DNA is exhaustively dialyzed into 0.01 M Tris HCl pH 7.5.

Reaction conditions for aminocaproic acid, bis (2-aminoethane) disulfide and 6-amino- α -methyl-D-manno (Example 5) are essentially identical.

Example 19

Coupling of Amino and Carboxy-Substituted Nucleic Acids to Proteins or Amines using 1-Ethyl-3-diisopropyl-aminocarbodiimide (EDAC).

Example of Streptavidin Coupled DNA

To a solution containing amino-or-carboxy-end substituted DNA (Example 11) (50 μ g) and 3H-labeled Streptavidin (50 μ g) dissolved in 1 ml 0.01M NaCl pH 7.5 (HCl) was added 5 mg EDAC. The reaction was incubated 20 hours in the dark and DNA was precipitated by addition of 4 M CaCl_2 (0.03 ml). DNA was redissolved in 1 ml water and this procedure was repeated 2 more times to remove unbound protein. The protein-coupled DNA was affinity purified in an iminobiotin-sepharose affinity column (K. Hoffman et al., PNAS 77:4666 1980) and dialyzed against 10 mM NaCl.

Example 20

N-Hydroxy Succinimide Activation of Carboxyhexyl-Substituted DNA and Coupling to Proteins or Amines.

Examples of Streptavidin Coupled DNA

Carboxy end substituted DNA (Example 19) (50 μ g) was converted to the triethylammonium salt by shaking an aqueous solution with Dowex 50-WX

(Et_3N^+). The solution was lyophilized and the dried DNA was dissolved in anhydrous dimethyl formamide (0.5 ml) to which was added dicyclohexylcarbodiimide (10.3 mg, 0.05 mol) and N-Hydroxysuccinimide (5.8 mg, 0.05 Mol). After incubating at room temperature for 20 hours the reaction was centrifuged and the supernatant dialyzed for 2 hours into 10 mM NaCl. Streptavidin (50 μ g) dissolved in 0.2 M borate, pH 8.5 (1 ml) was added to the N-hydroxysuccinimide activated DNA and the reaction was incubated 20 hours and dialyzed into 0.01M NaCl. The Streptavidin coupled DNA was purified by CaCl_2 precipitation and iminobiotin affinity chromatography as previously described.

Example 21

Reaction of DNA with Glyoxal

DNA (1 μ g) (BAM insert from plasmid pDK14) was dissolved in 0.025 M glyoxal (0.2 ml) and heated at 100°C for 30 minutes in a sealed tube. The reaction was dialyzed against 10 mM NaCl. In order to assess degree of reaction on guanosine, a portion of the reaction was subjected to acid depurination by lowering the pH to 1.0 with HCl, and heating at 100°C for 30 minutes. The depurinated DNA was removed by elution through DE-52 amino cellulose and the eluted purines analyzed by HPLC on reverse phase. Comparison of peak heights of adenosine, guanosine and glyoxal-guanosine adduct (R. Shapiro et al., Biochem 5:2799, 1966) revealed that 70% of the guanosines had been substituted.

Example 22

N-biotinyl-4-amino-acetophenone

To a solution of biotin-N-hydroxysuccinimide ester (50 μ g, 0.014 mmol) dissolved in 20 ml dimethylformamide (DMF) was added 4-aminophenylacetophenone (4.35 g, .03 mol) dissolved in 50 ml DMF and 100 ml 0.1 M borate buffer pH 8.5. After reaction at room temperature for 20 hours, the solvent was removed by rotary evaporation. The residual oil was triturated first with 0.1 N HCl and then with 5% sodium bicarbonate. The product was crystallized from ethanol.

Example 23

Biotinyl (4-aminophenyl)glyoxal

Selenium dioxide (0.75 g, 7 mmol) was dissolved in 4 ml dioxane containing 0.15 ml water. To this was added dropwise a solution of N-biotinyl-4-amino acetophenone (1.9 g, 7 mmol) dissolved in 5 ml dioxane. The reaction was refluxed for 3.5 hours after which

th mixtur was filtered and conc ntrated under vacuum. The crude product was purified by silica gel chromat graphy.

Example 24

1,N-Biotinyl-1,6-hexanediamine

Biotin-N-hydroxysuccinimide ester (1.0 g, 2.9 mmol) dissolved in dimethylformamide (5 μ m) was added to a solution of diaminohexane (1.15g, 10 mmol) in 0.1 M sodium borate (500 ml). After reacting at room temperature for 5 hours, the solution was rotary evaporated, redissolved in 10 ml water and chromatographed on Dowex 50 wx (H $+$). The column was washed with 50% aqueous methanol and the fraction eluted with triethylamine dissolved in 50% aqueous methanol (.3M) was collected. The evaporated residue was thoroughly triturated with ether and recrystallized from DMF/ether.

Example 25

CH₃-CO-CONH-(CH₂)₆-NH-Biotin

To a solution of pyruvic acid (.44 g, 5 mmol) cooled to 4°C, 20 ml anhydrous DMF was added isobutylchloroformated (0.64 g, 5 mmol) and tri-N-butylamine (1.43 g, 1.5 mmol). After 20 minutes at this temperature, an additional 1.4 g tri-N-butylamine was added and the mixture was added to a solution of 1,N-biotinyl-1,6-hexane diamine (Example 24) (.34 g, 1 mmol) dissolved in 30 ml DMF and 30 ml 0.1 M sodium borate. After reacting at 4°C for 1 hr. the mixture was allowed to stand at room temperature for an additional 20 hours and subsequently concentrated in vacuo. The mixture was purified by chromatography on silica gel and the product recrystallized from ethanol.

Example 26

CHO-CO-CONH-(CH₂)₆-NH-Biotin

Selenium dioxide (54 mg, 0.5 mmol) was dissolved in dioxane (0.5 ml) containing 25 μ l water. A solution of CH₃-CO-CONH-(CH₂)₆-NH-Biotin (Example 25) (0.21 g 0.5 mmol) dissolved in 1 ml dioxane was added dropwise and the reaction was heated at 100° for 4 hours. The resultant precipitate was removed by centrifugation, washed with dioxane and the supernatant was concentrated in vacuo. The crude mixture was chromatographed on silica gel.

Example 27

Reaction of DNA with Biotinylated Glyoxal Derivatives

5 DNA (1 μ g) as a triethylammonium salt was dissolved in 100 μ l water. To this was added 100 μ l of a solution of (N-biotinyl (4-aminophenyl)) glyoxal (0.05 M) or CHO-CO-CONH-(CH₂)₆-NH-Biotin (0.05M) in dimethylformamide. The mixture was heated at 100°C for 30 minutes in a sealed tube and subsequently dialyzed against 0.01 M NaCl. Extent of reaction was determined by acid depurification and HPLC assay of purine nucleosides, as described previously.

Example 28

Bromination of pBR 322 DNA

20 To a solution of pBR 322 DNA (100 μ g) dissolved in 0.5 M acetate buffer pH 5.5. was added bromine (5.5 μ l, 0.1 mmol). The reaction was incubated at 60° for 30 hours and exhaustively dialyzed against 0.01 M NaCl.

Example 29

Reaction of Brominated pBR 322 DNA with Thiols.

30 Example of Reaction with Cysteamine and 3-Mercaptopropionic acid.

35 A solution of brominated pBR 322 DNA (500 μ g) in 0.1 M borate pH 8.5 (1 ml) was incubated with either cysteamine or 3-mercaptopropionic acid (20 mg) for 20 hours at room temperature under an argon atmosphere. The resultant amine-or carboxy- substituted DNA was dialyzed against 0.01 M NaCl.

Example 30

Reaction of Brominated pBR322 DNA with Amines.

Example of Reaction with 1,6-Diaminohexane

45 A solution of brominated pBR 322 DNA (100 μ g) and 1 M of 1,6-diaminohexane (1ml) were heated at 65° for 3 hours under an argon atmosphere. The resultant amine-substituted DNA was dialyzed against 0.1 M NaCl.

Example 31

55 Synthesis of a Protein Coupled to a Signal Generating Polynucleotide. Example of IgG Coupled to Chemically Radio-labeled DNA.

Fd DNA was end labeled with UMP using termi-

nal transfr rase under conditions described in Example 11. The end labelled DNA was derivatized with 2,4,5-trichloroanilin (Exampl 1) and reacted with DCTA-SH under identical conditions to those described in Example 1a. The end labelled, DCTA derivatized DNA was oxidized with sodium periodate, reacted with cysteamine and reduced with sodium borohydride as described in Example 12. This was in turn reacted with bromoacetylated IgG (Example 14) using conditions described in Example 15.

Example 32

Use of Bacteriophage M13 as Bridging Entity

Using techniques of recombinant DNA technology, an asymmetric DNA sequence can be inserted in the replicative (double-stranded form) of a single-stranded phage such as M13. One strand of the insert will be deficient in guanine residues. As a result of this insertion, two single-stranded phages will be obtained in both polarities, one containing the (G-) strand, i.e., no guanylate residues of the asymmetric sequence, the other containing the sequence complementary to the G(-) sequence, to be called the G(+) sequence.

The G(+) phage is used as the vector (bridging entity) for carrying a DNA probe of interest such as, for example, herpes simplex virus I DNA sequences.

The G(-) phage (signalling entity) is chemically reacted with a guanosine specific reagent, such as a 1,2-dicarbonyl reagent. The G(-) insert in the G(-) phage would not be modified because it lacks guanylate residues.

A general protocol for the preparation of single-stranded M13 would be as follows:

1. M13 mp8 rf (replicative form, double-stranded), is grown. It is cut with Hinc II, which leaves blunt ends.
2. pd(G-T)₅ and pd(A-C)₅ are provided and hybridized to form a perfect double-strand. The ends must be perfectly matched. In order to obtain this condition it is necessary to use high C_{ot} conditions for hybridization.
3. The hybrids are ligated in the presence of the restriction enzyme Rsa I. Rsa I recognizes the sequence GTAC, and hence will cut to leave blunt ends and proofread the ligation.
4. The ligation products are isolated. They are double-stranded poly d (G-T) poly d (A-C). (Any complementary, repeating, low complexity sequence can be used. The subsequent modification and chemistries must be adjust d accordingly.)
5. Alkaline phosphatase is used to r mov 5' phosphat s.
6. Polynucleotide kinas and ^{32P}-ATP ar used to replace 5' nds with ^{32P}-phosphat s.

5. Th reaction mixtur is run over 15-20% non-d naturing polyacrylamide gels to s parate the different size fragments.
6. Th fragm nts are located on gels by autoradiography.
7. The desired size bands are eluted out of the gel by cutting, mashing and then extracting the gel with high salt buffer. Fragments having 50-100 bp or larger are preferred.
10. DNA is concentrated by any of a numb r of possible techniques, such as ethanol precipitation, spermine precipitation, lyophilization, or the like.
11. The fragments are ready to be cloned into Hinc II-cut M13.
15. Using the standard cloning technique, the following sequence is performed:
 - a. Fragments are ligated into M13;
 - b. Cells (for example E. coli JM103) are transformed with the M13;
 - c. Transformed cells are plated; and
 - d. Recombinants are selected.
20. There are two possible routes for selection of recombinants:
 - a. If a known size class has been inserted, plaques will be picked and sequenced to ch ck for the presence of insert.
 - b. An alternative procedure is to shotgun all the sequences made in step 4 into M13. This protocol requires that many more clones b picked and then checked by sequencing.
25. Once a suitable clone has been obtained (M13 with the appropriate size sequence), the strand that gives GTGT, etc., in the single-strand replicating form will be selected. This clone is then used for further genetic engineering by inserting sequences from a variety of pathogens into the replicating form.
30. The strand that gives ACAC etc. in the replicating form is cloned in mass culture and chemically modified with a reporter (signal generation portion) that is specific for G's. Thus the G(-) phage is exhaustively reacted with the bifunctional reagent p-azidophenyl glyoxal (APG). The dicarbonyl moiety of APG reacts only with guanosine residues in single-stranded portions of th DNA. The insert lacking guanosine is not affected by this treatment.
35. The (G+) and derivatized (G-) DNAs are mixed in equimolar concentration and allowed to hybridize to target DNAs and to each other. Visualization of the hybrids is by standard signal reporting techniques.
- 40.
- 45.
- 50.
- 55.

Claims

1. A method of detecting in a sampl an analyte hav-

ing a molecularly recognizable portion thereon, comprising:

forming a complex comprising (1) said analyte bound to (2) a molecular bridging entity comprising a portion capable of recognizing and binding to said molecularly recognizable analyte portion, and a portion comprising a polynucleotide sequence; and (3) a signalling entity comprising a recognition portion capable of complexing to said polynucleotide portion of said bridging entity, and a signal generating portion with the proviso that when said analyte is a polynucleotide, the signal generating portion is not an enzyme that is covalently linked to said recognition portion; and
detecting a signal provided by said signal generating portion present in said complex.

2. The method according to Claim 1, characterized in that said analyte is immobilized.
3. The method according to Claim 1, characterized in that said analyte is a microorganism or fragment thereof which is selected from the group consisting of a virus, a viral component, a bacterium, a bacterial component, a cell, a cellular component and a pathogen or non-pathogen or a component thereof.
4. The method according to Claim 3, characterized in that said molecularly recognizable portion on said microorganism is selected from the group consisting of an RNA and DNA nucleotide, oligonucleotide sequence, polynucleotide sequence, peptide, polypeptide protein, antigen, antibody, lectin, saccharide, hormone, ligand, enzyme inhibitor, enzyme cofactor, enzyme, and substrates and receptors thereof.
5. The method according to Claim 1, characterized in that said bridging entity recognizing portion is selected from the group consisting of an RNA or DNA nucleotide, an oligonucleotide sequence, polynucleotide sequence, peptide, polypeptide, and protein, and an antigen, a polyclonal antibody, a monoclonal antibody, a saccharide, a lectin, a hormone, a ligand, an enzyme inhibitor, an enzyme cofactor, an enzyme, and substrates and receptors thereof.
6. The method according to Claim 1, characterized in that said bridging entity polynucleotide sequence is selected from the group consisting of a poly deoxy-G, poly deoxy-C, poly deoxy-T or poly deoxy-A sequence, or any poly-ribo or -deoxyribo purine, pyrimidine analog, a gene product or fragment thereof, or a sequence substantially comprising adenine residues, a sequence

substantially comprising a residue selected from the group consisting of guanine, cytosine, thymine, uracil, guanosine .

- 5 7. The method of claim 1 characterized in that said bridging entity recognizing portion is covalently bound to said bridging entity polynucleotide sequence.
- 10 8. The method according to Claim 1, characterized in that said bridging entity is selected from the group consisting of a single-stranded, double stranded or partially double-stranded circular DNA polymer, a circular DNA polymer derived from a filamentous phage, an M13 phage or a variant thereof, a DNA molecule comprising a polynucleotide sequence complementary to part or all of a gene sequence of a nucleic acid-containing organism.
- 15 9. The method according to Claim 1, characterized in that said signalling entity receptor is a polynucleotide sequence selected from the group comprising a poly deoxy C, poly deoxy G, poly deoxy A, poly deoxy T sequence, a repeating sequence of low complexity, a sequence portion substantially comprising cytosine residues or guanosine residues, and a sequence coding for a gene product or fragment thereof.
- 20 10. The method according to Claim 1, characterized in that said signalling entity is selected from the group consisting of a single stranded, double stranded, or partially double-stranded polynucleotide polymer, a modified naturally occurring DNA, a polynucleotide polymer derived from a T (even) phage, a modified DNA carrying a cloned insert, a polymer derived from a filamentous phage, M13 phage or a variant thereof, and a polymer derived from a circular DNA molecule covalently attached to a non radio-labelled signal generating moiety.
- 25 11. The method according to Claim 1, characterized in that said signal generating portion is selected from the group consisting of a radioactive moiety, an enzyme, a lectin, an antibody, an antigen, a biotin moiety, a saccharide, a fluorogenic compound, an electron dense compound, a polynucleotide sequence capable of recognizing a signal-containing moiety, a compound capable of binding to an insoluble phase, a latex particle, a resin and a bacterium.
- 30 12. The method according to Claim 1, characterized in that said detecting step is selected from the group consisting of a radioactivity measurement, an enzymatic reaction, a fluorescence measure-

ment, an electron microscopic measurement, an antibody/antigen complexation reaction, a biotin and biotin binding moiety complexation reaction, an avidin-streptavidin complexation reaction, an electron density measurement, a saccharide and lectin complexation reaction, a binding step on an insoluble phase, and complexation between a signalling entity comprising a cloned insert on a naturally occurring modified DNA, and the bridging moiety, followed by binding a modified lectin to said signalling entity.

13. A polynucleotide sequence covalently attached to an antibody. 15

14. The sequence of Claim 13 wherein said antibody is monoclonal.

15. A polynucleotide sequence covalently attached to a lectin. 20

16. A polynucleotide sequence covalently attached to a saccharide having up to 20 saccharide units.

17. A polynucleotide sequence covalently attached to receptor. 25

18. A polynucleotide sequence covalently attached to a hormone.

19. A kit useful for the detection of an analyte (A) having a molecularly recognizable portion thereof, comprising:

- I) a carrier being compartmentalized to receive in close confinement therein therein one or more container means;
- II) a first container means containing a molecular bridging entity (B) having thereon:
 - (i) a portion capable of recognizing said molecularly recognizable portion on said analyte (A); and
 - (ii) a portion comprising a polynucleotide sequence;
- III) a second container means containing a signalling entity (C) having thereon:
 - (i) a polynucleotide portion capable of annealing to said polynucleotide portion of said bridging entity (B) thereby to form a stable polynucleotide hybrid; and
 - (ii) a signal generating portion with the proviso that when said analyte is a polynucleotide, the signal generating portion is not an enzyme that is covalently linked to said recognition portion; and
- IV) other container means, where appropriate, containing molecular entities necessary or useful in detecting, making detectable or enhancing the signal of the said signal generat-

ing portion of said signalling entity (C).

20. The method according to claim 11 wherein said fluorogenic compound is directly or indirectly linked to said signalling entity. 5

21. The method according to claims 1 or 2, characterized in that said forming step comprises contacting said analyte with said molecular bridging entity to form a first complex and thereafter contacting said first complex with said signalling entity to form a subsequent complex.

22. The method according to claims 1 or 2, characterized in that said forming step comprises contacting said molecular bridging entity with said signalling entity to form a first complex and thereafter contacting said first complex with said analyte to form a subsequent complex.

23. The method according to claim 2 further comprising a washing step after formation of the first complex, or after formation of the subsequent complex, or after each complex formation. 30

Patentansprüche

1. Verfahren zum Nachweis eines Analyten mit einem erkennbaren Molekülanteil in einer Probe, umfassend:
Bildung eines Komplexes, umfassend (1) den Analyten, der an (2) eine molekulare brückenbildende Einheit gebunden ist, die einen Anteil umfaßt, der den erkennbaren Molekülanteil des Analyten erkennen und binden kann, und einen Anteil, der eine Polynucleotidsequenz umfaßt; und (3) einen signalgebenden Anteil, der einen Erkennungsanteil umfaßt, der mit dem Polynucleotidanteil der brückenbildenden Einheit einen Komplex bilden kann, und einen signalerzeugenden Anteil, mit der Maßgabe, daß, falls der Analyt ein Polynucleotid ist, der signalerzeugende Anteil kein Enzym ist, das kovalent an den Erkennungsanteil gebunden ist; und
Nachweis eines Signals, das von dem in dem Komplex vorhandenen signalerzeugenden Anteil geliefert wird.
2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß der Analyt immobilisiert ist.
3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß der Analyt ein Mikroorganismus oder ein Fragment davon ist, ausgewählt aus einem Virus, in dem viralen Bestandteil, einem Bakterium, einem bakteriellen Bestandteil, in einer Zelle, einem zellulären Bestandteil und einem Pa-

thogen oder Nicht-Pathogen oder ein m Be- standteil davon.

4. Verfahren nach Anspruch 3, dadurch gekennzeichnet, daß der erkennbare Mol külanteil auf dem Mi- kroorganismus ausgewählt ist aus einem RNA- und DNA-Nucleotid, einer Oligonucleotidsequenz, Po- lynucleotidsequenz, einem Peptid, Polypeptid, Protein, Antigen, Antikörper, Lectin, Saccharid, Hormon, Liganden, Enzyminhibitor, Enzymcofak- tor, Enzym und Substraten und Rezeptoren da- von.

5. Verfahren nach Anspruch 1, dadurch gekenn- zeichnet, daß der die brückenbildende Einheit er- kennende Anteil ausgewählt ist aus einem RNA- oder DNA-Nucleotid, einer Oligonucleotidsequenz, Po- lynucleotidsequenz, einem Peptid, Polypeptid, Protein, Antigen, polyclonalen Antikörper, monoklonalen Antikörper, Saccharid, Lectin, Hormon, Liganden, Enzyminhibitor, Enzymcofak- tor, Enzym und Substraten und Rezeptoren da- von.

6. Verfahren nach Anspruch 1, dadurch gekenn- zeichnet, daß die Polynucleotidsequenz der brückenbildenden Einheit ausgewählt ist aus ei- ner Polydesoxy-G-, Polydesoxy-C-, Polydesoxy- T- oder Polydesoxy-A-Sequenz, oder einem be- liebigen Polyribo- oder -desoxyribopurin oder -pyrimidin oder Analogen, einem Genprodukt oder Fragment davon, einer Sequenz, die im wesent- lichen Adeninreste umfaßt, und einer Sequenz, die im wesentlichen Reste umfaßt, ausgewählt aus Guanin, Cytosin, Thymin, Uracil oder Guanosin.

7. Verfahren nach Anspruch 1, dadurch gekenn- zeichnet, daß der die brückenbildende Einheit er- kennende Anteil kovalent an die Polynucleotidse- quenz der brückenbildenden Einheit gebunden ist.

8. Verfahren nach Anspruch 1, dadurch gekenn- zeichnet, daß die brückenbildende Einheit aus- gewählt ist aus einem einzelsträngigen, doppelsträngigen oder teilweise doppelsträngigen zirkulären DNA-Polymer, einem zirkulären DNA- Polymer, das von einem filamentösen Phagen, einem M13-Phagen oder einer Variante davon stammt, einem DNA-Molekül, das eine Polynu- cleotidsequenz umfaßt, die zu einem Teil einer G nsequenz oder iner gesamt n Gensequenz ein s eine Nucleinsäure enthaltend n Organis- mus komplem ntär ist.

9. Verfahren nach Anspruch 1, dadurch gekenn- z ichnet, daß der di signalgebende Einheit er-

5 k nnende Anteil ein Polynucleotidsequenz ist, ausgewählt aus einer Polydesoxy-C-, Polydesoxy- G-, Polyd soxy-A-, Polydesoxy-T-Sequenz, ei- ner sich wiederholenden Sequenz geringer Kom- plexität, ein m Sequenzanteil, der im wesentli- chen Cytosinreste oder Guanosinreste umfaßt, und einer Sequenz, die ein Genprodukt oder Fragment davon codiert.

10. Verfahren nach Anspruch 1, dadurch gekenn- zeichnet, daß der die signalgebende Einheit er- kennende Anteil ausgewählt ist aus einem einzel- strängigen, doppelsträngigen oder teilweis dop- pelsträngigen Polynucleotidpolymer, einer modifi- zierten, natürlich vorkommenden DNA, ein m Polynucleotidpolymer, das von einem (geradzahligen) T-Phagen stammt, einer modifizierten DNA, die eine clonierte Insertion trägt, einem Po- lymer, das von einem filamentösen Phag n, M13-Phagen oder einer Variante davon stammt, und einem Polymer, das von einem zirkulären DNA-Molekül stammt, das kovalent an ein nicht- radioaktiv markierte signalerzeugende Einheit geknüpft ist.

15

20

25

30

35

40

45

50

55

60

65

70

75

80

85

90

95

100

105

110

115

120

125

130

135

140

145

150

155

160

165

170

175

180

185

190

195

200

205

210

215

220

225

230

235

240

245

250

255

260

265

270

275

280

285

290

295

300

305

310

315

320

325

330

335

340

345

350

355

360

365

370

375

380

385

390

395

400

405

410

415

420

425

430

435

440

445

450

455

460

465

470

475

480

485

490

495

500

505

510

515

520

525

530

535

540

545

550

555

560

565

570

575

580

585

590

595

600

605

610

615

620

625

630

635

640

645

650

655

660

665

670

675

680

685

690

695

700

705

710

715

720

725

730

735

740

745

750

755

760

765

770

775

780

785

790

795

800

805

810

815

820

825

830

835

840

845

850

855

860

865

870

875

880

885

890

895

900

905

910

915

920

925

930

935

940

945

950

955

960

965

970

975

980

985

990

995

1000

1005

1010

1015

1020

1025

1030

1035

1040

1045

1050

1055

1060

1065

1070

1075

1080

1085

1090

1095

1100

1105

1110

1115

1120

1125

1130

1135

1140

1145

1150

1155

1160

1165

1170

1175

1180

1185

1190

1195

1200

1205

1210

1215

1220

1225

1230

1235

1240

1245

1250

1255

1260

1265

1270

1275

1280

1285

1290

1295

1300

1305

1310

1315

1320

1325

1330

1335

1340

1345

1350

1355

1360

1365

1370

1375

1380

1385

1390

1395

1400

1405

1410

1415

1420

1425

1430

1435

1440

1445

1450

1455

1460

1465

1470

1475

1480

1485

1490

1495

1500

1505

1510

1515

1520

1525

1530

1535

1540

1545

1550

1555

1560

1565

1570

1575

1580

1585

1590

1595

1600

1605

1610

1615

1620

1625

1630

1635

1640

1645

1650

1655

1660

1665

1670

1675

1680

1685

1690

1695

1700

1705

1710

1715

1720

1725

1730

1735

1740

1745

1750

1755

1760

1765

1770

1775

1780

1785

1790

1795

1800

1805

1810

1815

1820

1825

1830

1835

1840

1845

1850

1855

1860

1865

1870

1875

1880

1885

1890

1895

1900

1905

1910

1915

1920

1925

1930

1935

1940

1945

1950

1955

1960

1965

1970

1975

1980

1985

1990

1995

2000

2005

2010

2015

2020

2025

2030

2035

2040

2045

2050

2055

2060

2065

2070

2075

2080

2085

2090

2095

2100

2105

2110

2115

2120

2125

2130

2135

2140

2145

2150

2155

2160

2165

2170

2175

2180

2185

2190

2195

2200

2205

2210

2215

2220

2225

2230

2235

2240

2245

2250

2255

2260

2265

2270

2275

2280

2285

2290

2295

2300

2305

2310

2315

2320

2325

2330

2335

2340

2345

2350

2355

2360

2365

2370

2375

2380

2385

2390

2395

2400

2405

2410

2415

2420

2425

2430

2435

2440

2445

2450

2455

2460

2465

2470

2475

2480

2485

2490

2495

2500

2505

2510

2515

2520

2525

2530

2535

2540

2545

2550

2555

2560

2565

2570

2575

2580

2585

2590

2595

2600

2605

2610

2615

2620

2625

2630

2635

2640

2645

2650

2655

2660

2665

2670

2675

2680

2685

2690

2695

2700

2705

2710

2715

2720

2725

2730

2735

2740

2745

2750

2755

2760

2765

2770

2775

2780

2785

2790

2795

2800

2805

2810

2815

2820

2825

2830

2835

2840

2845

2850

2855

2860

2865

2870

2875

2880

2885

2890

2895

2900

2905

2910

2915

2920

2925

2930

2935

2940

2945

2950

2955

2960

2965

2970

2975

2980

2985

2990

2995

3000

3005

3010

3015

3020

3025

3030

3035

3040

3045

3050

3055

3060

3065

3070

3075

3080

3085

3090

3095

3100

3105

3110

3115

3120

3125

3130

3135

3140

3145

3150

3155

3160

3165

3170

3175

3180

3185

3190

3195

3200

3205

3210

3215

3220

3225

3230

3235

3240

3245

3250

3255

3260

3265

3270

3275

3280

3285

3290

3295

3300

3305

3310

3315

3320

3325

3330

3335

3340

3345

3350

3355

3360

3365

3370

3375

3380

3385

3390

3395

3400

3405

3410

3415

3420

3425

3430

3435

3440

3445

3450

3455

3460

3465

3470

3475

3480

3485

3490

3495

3500

3505

3510

3515

3520

3525

3530

3535

3540

3545

3550

3555

3560

3565

3570

3575

3580

3585

3590

3595

3600

3605

3610

3615

3620

3625

3630

3635

3640

3645

3650

3655

3660

3665

3670

3675

3680

3685

3690

3695

3700

3705

3710

3715

3720

3725

3730

3735

3740

3745

3750

3755

3760

3765

3770

3775

3780

3785

3790

3795

3800

3805

3810

3815

3820

3825

3830

3835

3840

3845

3850

3855

3860

3865

3870

3875

3880

3885

3890

3895

3900

3905

3910

3915

3920

3925

3930

3935

3940

3945

3950

3955

3960

3965

3970

3975

3980

3985

3990

3995

4000

4005

4010

4015

4020

4025

4030

4035

4040

4045

4050

4055

4060

4065

4070

4075

4080

4085

4090

4095

4100

4105

4110

4115

4120

4125

4130

4135

4140

4145

4150

4155

4160

4165

4170

4175

4180

4185

4190

4195

4200

4205

4210

4215

4220

4225

4230

4235

4240

4245

4250

4255

4260

4265

4270

4275

4280

4285

4290

4295

4300

4305

4310

4315

4320

4325

4330

4335

4340

4345

4350

4355

4360

4365

4370

4375

4380

4385

4390

4395

4400

4405

4410

4415

4420

4425

4430

4435

4440

4445

4450

4455

4460

4465

4470

4475

4480

4485

4490

4495

4500

4505

4510

4515

4520

4525

4530

4535

4540

4545

4550

4555

4560

4565

4570

4575

4580

4585

4590

4595

4600

4605

4610

4615

4620

4625

4630

4635

4640

4645

4650

4655

4660

4665

4670

4675

4680

4685

4690

4695

4700

4705

4710

4715

4720

4725

4730

4735

4740

4745

4750

4755

4760

4765

4770

4775

4780

4785

4790

4795

4800

4805

4810

4815

4820

4825

4830

4835

4840

4845

4850

4855

4860

4865

4870

4875

4880

4885

4890

4895

4900

4905

4910

4915

4920

4925

4930

4935

4940

4945

4950

4955

4960

4965

4970

4975

4980

4985

4990

4995

5000

5005

5010

5015

5020

5025

5030

5035

5040

5045

5050

5055

5060

5065

5070

5075

5080

5085

5090

5095

5100

5105

5110

5115

5120

5125

5130

5135

5140

5145

5150

5155

5160

5165

5170

5175

5180

5185

5190

5195

5200

5205

5210

5215

5220

5225

5230

5235

5240

5245

5250

5255

5260

5265

5270

5275

5280

5285

5290

5295

5300

5305

5310

5315

5320

5325

5330

5335

5340

5345

5350

5355

5360

5365

5370

5375

5380

5385

5390

5395

5400

5405

5410

5415

5420

5425

5430

5435

5440

5445

5450

5455

5460

5465

5470

5475

5480

5485

5490

5495

5500

5505

5510

5515

5520

5525

5530

5535

5540

5545

5550

5555

5560

5565

5570

5575

5580

5585

5590

5595

5600

5605

5610

5615

5620

5625

5630

5635

5640

5645

5650

5655

5660

5665

5670

5675

5680

5685

5690

5695

5700

5705

5710

5715

5720

5725

5730

5735

5740

5745

5750

5755

5760

5765

5770

5775

5780

5785

5790

5795

5800

5805

5810

5815

5820

5825

5830

5835

5840

5845

5850

5855

5860

5865

5870

5875

5880

5885

5890

5895

5900

5905

5910

5915

5920

5925

5930

5935

5940

5945

5950

5955

5960

5965

5970

5975

5980

5985

5990

5995

6000

6005

6010

6015

6020

6025

6030

6035

6040

6045

6050

6055

6060

6065

6070

6075

6080

6085

6090

6095

6100

6105

6110

6115

6120

6125

6130

6135

6140

6145

6150

6155

6160

6165

6170

6175

6180

6185

6190

6195

6200

6205

6210

6215

6220

6225

6230

6235

6240

6245

6250

6255

6260

6265

6270

6275

6280

6285

6290

6295

6300

6305

6310

6315

6320

6325

6330

6335

6340

6345

6350

6355

6360

6365

6370

6375

6380

6385

6390

6395

6400

6405

6410</

Antikörper verknüpft ist.

14. S qu nz nach Anspruch 13, wobei der Antikörper ein monoklonaler Antikörper ist.

15. Polynukleotidsequenz, die kovalent mit einem Lectin verknüpft ist.

16. Polynukleotidsequenz, die kovalent mit einem Saccharid mit bis zu 20 Saccharideinheiten verknüpft ist.

17. Polynukleotidsequenz, die kovalent mit einem Rezeptor verknüpft ist.

18. Polynukleotidsequenz, die kovalent mit einem Hormon verknüpft ist.

19. Kit, verwendbar zum Nachweis eines Analyten (A) mit einem erkennbaren Molekülanteil, umfassend:

- I) einen Träger, der zur Aufnahme von einem oder mehreren Behältern auf engem Raum aufgeteilt ist;
- II) einen ersten Behälter, der eine molekulare brückenbildende Einheit (B) enthält, die
 - (i) einen Anteil, der den erkennbaren Molekülanteil auf dem Analyten (A) erkennen kann; und
 - (ii) einen eine Polynukleotidsequenz umfassenden Anteil umfaßt;
- III) einen zweiten Behälter, der eine signalgebende Einheit (C) enthält, die
 - (i) einen Polynukleotidanteil, der den Polynukleotidanteil der brückenbildenden Einheit (B) anlagern kann, wodurch ein stabiles Polynukleotidhybrid gebildet wird; und
 - (ii) einen signalerzeugenden Anteil umfaßt, mit der Maßgabe, daß, falls der Analyt ein Polynukleotid ist, der signalerzeugende Anteil kein Enzym ist, das kovalent an den Erkennungsanteil gebunden ist; und
- IV) falls erforderlich weitere Behälter, die molekulare Einheiten enthalten, die notwendig oder nützlich sind, das Signal des signalerzeugenden Anteils der signalgebenden Einheit (C) nachzuweisen, nachweisbar zu machen oder zu erhöhen.

20. Verfahren nach Anspruch 11, wobei die fluorogene Verbindung direkt oder indirekt mit der signalgebenden Einheit verbunden ist.

21. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß der Schritt der Bildung des Inkontaktbringens des Analyten mit der molekularen brückenbildenden Einheit zur Bildung eines ersten Komplexes umfaßt und im Anschluß daran das Inkontaktbringen der signalgebenden Einheit zur Bildung eines weiteren Komplexes.

22. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß der Schritt der Bildung des Inkontaktbringens der molekularen brückenbildenden Einheit mit der signalgebenden Einheit zur Bildung eines ersten Komplexes umfaßt und im Anschluß daran das Inkontaktbringen des ersten Komplexes mit dem Analyten zur Bildung eines weiteren Komplexes.

23. Verfahren nach Anspruch 2, außerdem umfassend einen Waschschritt nach der Bildung des ersten Komplexes oder nach der Bildung des weiteren Komplexes oder nach jeder Komplexbildung.

Revendications

1. Procédé de détection dans un échantillon d'un analyte contenant une portion reconnaissable du point de vue moléculaire, comprenant les étapes de :
 - formation d'un complexe comprenant (1) ledit analyte lié à (2) une entité moléculaire formant un pont constituée d'une portion capable de reconnaître à la portion d'analyte reconnaissable du point de vue moléculaire et de s'y lier, et d'une portion constituée d'une séquence polynucléotidique ; et (3) une entité produisant un signal, constituée d'une portion de reconnaissance capable de se complexer à ladite portion polynucléotidique de ladite entité formant un pont, et une portion générant un signal à la condition que, lorsque ledit analyte est un polynucléotide, la portion générant un signal ne soit pas une enzyme liée par covalence à ladite portion de reconnaissance ; et
 - détection d'un signal émis par ladite portion générant un signal présente dans ledit complexe.
2. Procédé selon la revendication 1, caractérisé en ce que ledit analyte est immobilisé.
3. Procédé selon la revendication 1, caractérisé en ce que ledit analyte est un micro-organisme ou un fragment de celui-ci, qui est choisi dans le groupe constitué d'un virus, d'un composant viral, d'une bactérie, d'un composant bactérien, d'un cellule, d'un composant cellulaire et d'un agent pathogène ou non pathogène ou d'un composant de celui-ci.
4. Procédé selon la revendication 3, caractérisé en

15. Séquence polynucléotidique fixé par covalence sur un ligandin .

16. Séquence polynucléotidique fixée par covalence à un saccharide ayant jusqu'à 20 unités saccharidiques.

17. Séquence polynucléotidique fixée par covalence sur un récepteur.

18. Séquence polynucléotidique fixée par covalence sur une hormone.

19. Kit pouvant être utilisé pour la détection d'un analyte (A) ayant une portion reconnaissable du point de vue moléculaire, comprenant :

- I) un support séparé en compartiments pour recevoir, étroitement renfermés à l'intérieur, un ou plusieurs dispositifs de conditionnement ;
- II) un premier dispositif de conditionnement contenant une entité moléculaire formant un pont (B) contenant :

 - (i) une portion capable de reconnaître ladite portion reconnaissable du point de vue moléculaire sur ledit analyte (A) ; et
 - (ii) une portion constituée d'une séquence polynucléotidique ;

- III) un second dispositif de conditionnement contenant une entité signal (C) ayant sur elle :

 - (i) une portion polynucléotidique capable de s'hybrider à ladite portion polynucléotidique de ladite entité formant un pont (B) pour former ainsi un hybride polynucléotidique stable ; et
 - (ii) une portion générant un signal, à la condition que, quand ledit analyte est un polynucléotide, la portion générant un signal ne soit pas une enzyme liée par covalence à ladite portion de reconnaissance ; et

- IV) d'autres moyens de conditionnement, si nécessaire, contenant les entités moléculaires nécessaires ou utiles pour détecter, rendre détectable ou augmenter le signal de ladite portion générant un signal de ladite entité produisant un signal (C).

20. Procédé selon la revendication 11, dans lequel ledit composé fluorogène est directement ou indirectement lié à ladite entité produisant un signal.

21. Procédé selon les revendications 1 ou 2, caractérisé en ce que ladite étape de formation comprend la mise en contact dudit analyte avec ladite entité moléculaire formant un pont, pour former un premier complexe, puis la mise en contact dudit premier complexe avec ladite entité produisant un signal pour former un second complexe.

22. Procédé selon les revendications 1 ou 2, caractérisé en ce que ladite étape de formation comprend la mise en contact de ladite entité moléculaire formant un pont avec ladite entité produisant un signal pour former un premier complexe, puis la mise en contact dudit premier complexe avec ledit analyte pour former un second complexe.

23. Procédé selon la revendication 2, comprenant en outre une étape de lavage après la formation du premier complexe, ou après la formation du second complexe, ou après la formation de chacun des complexes.

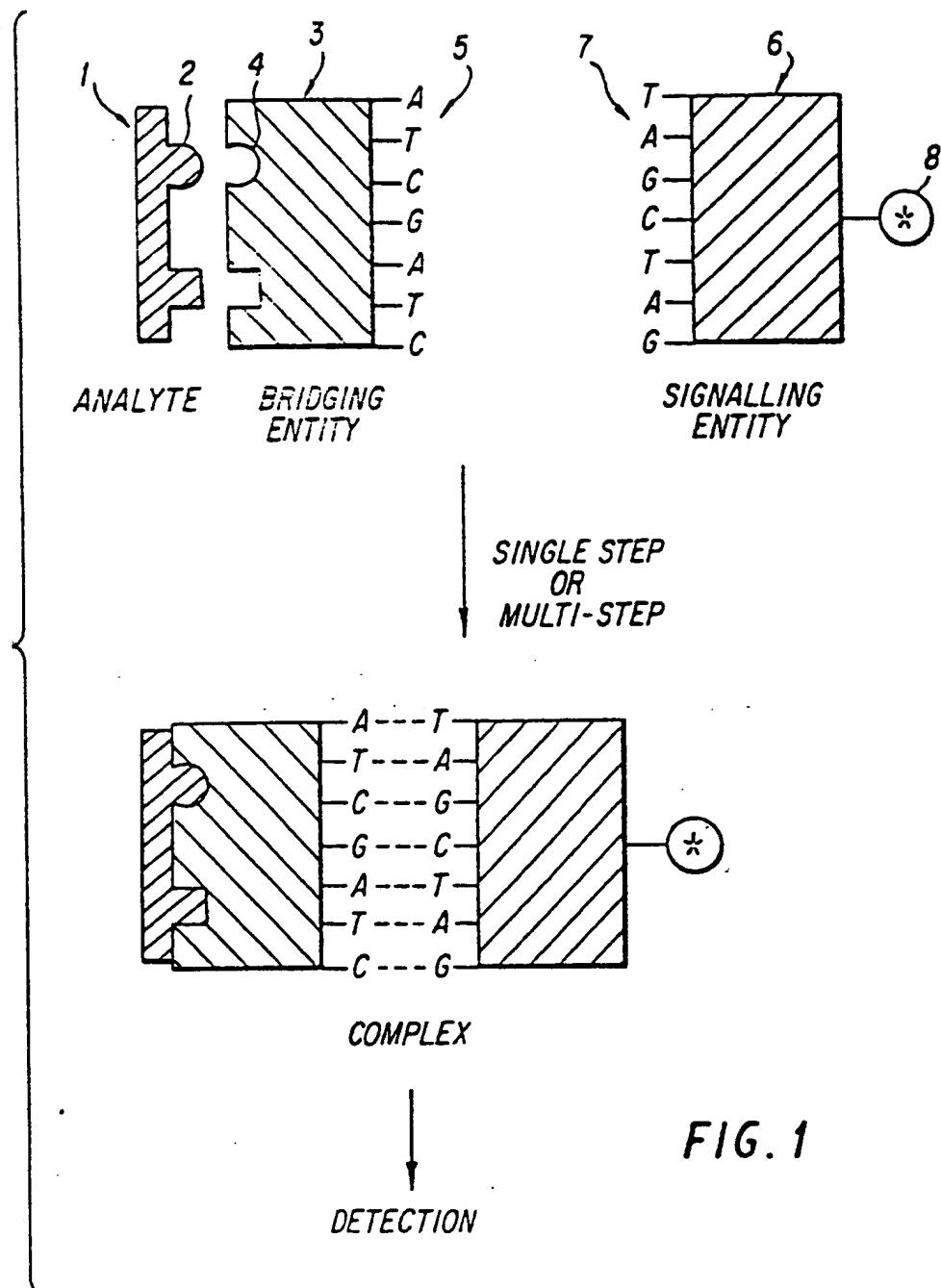


FIG. 1

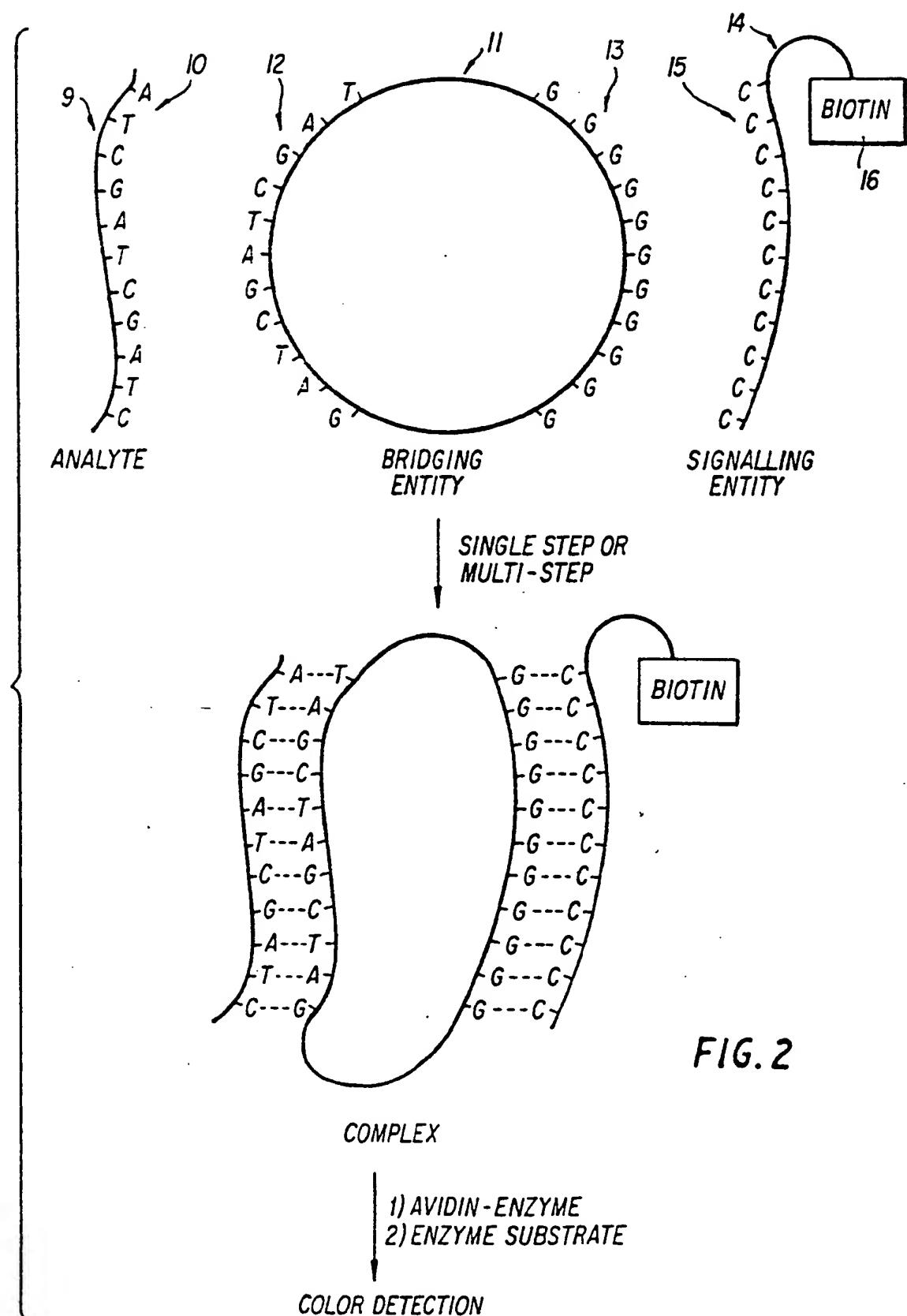


FIG. 2